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Immune-stimulatory potential of transgenic CD40L expressing dendritic cells

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Content

Summary

Dendritic cells (DCs) as the professional representatives of antigen presenting cells (APCs) play a pivotal role in stimulating the immune system. They present processed antigens bound to major histocompatibility complex (MHC) class I or II to $CD8^+$ cytolytic T cells (CTLs) or $CD4^+$ T cells, respectively. In an immature state DCs (immature DCs, iDCs) are found resting in peripheral tissues where they induce tolerance against self-antigens. iDCs become activated by exposure to pathogen associated molecular patterns (PAMPs) or inflammatory cytokines and shift from a tolerance inducing to an immune activating phenotype. A unique function of DCs is that they can cross-present exogenous antigen to CTLs. This signal triggered by CD40L, expressed on activated $CD4^+$ T helper cells, is called licensing signal. In tumour immunology the activation of tumour antigen-specific CTLs is an important step toward tumour rejection.

Dendritic cells (DC) matured *in vitro* by Toll-like receptor (TLR)-4 engagement with lipopolysaccharide (LPS) show a dynamic phenotype, initially conferring immune stimulation followed by immune regulation/suppression. This is indicated by an early secretion of interleukine (IL)- 12, the key cytokine for T helper type 1 (Th1) polarisation and CTL activation, followed by release of the immune regulatory cytokine IL-10. In preliminary experiments it was demonstrated, using DNA micro array technology, that these phenotypic and functional characteristics may be modulated by CD40/CD40L signalling. We investigated the effect of lentiviral transfer of CD40L or GFP control into DCs 6 hours (semi matured, smDC) or 48 hours (fully matured, mDC) after maturation with LPS/IFN-γ. Mature DCs that had exhausted their capacity for cytokine secretion were re-induced to secrete IL-12 upon CD40L but not GFP gene transfer. Importantly, IL-10 secretion remained undetectable. In allogeneic mixed leukocyte reactions (alloMLR), mDCs expressing CD40L triggered strong $CD8^+$ T-cell proliferation accompanied by an accumulation of Granzyme (GrB), a marker for cytotoxic activity. From these data we conclude that CD40L signalling rescues an immune stimulatory Th1 DC phenotype in TLR-4 pre-activated mDCs. These findings may impact on the rational design of DC-based cancer vaccines.

Zusammenfassung

Die Aktivität des Immunsystems wird von dendritischen Zellen (Dendritic Cells, DCs) reguliert, die eine zentrale Position an der Schaltstelle zwischen angeborener und erworbener Immunität einnehmen. DCs befinden sich in ihrer unreifen Form in allen Geweben wo sie Selbstantigene prozessieren und gegen diese Toleranz aufrechterhalten. Wurde jedoch ein Ausreifungsprozess in den DCs durch die Einwirkung von entzündlichen Zytokinen oder durch das Erkennen von Pathogen assoziierten molekularen Mustern (pathogen associated molecular patterns, PAMPs) in Gang gesetzt verschiebt sich ihre Funktion von Toleranzauslösung zu Immunaktivierung. Dabei verstärkt sich die Fähigkeit von DCs prozessiertes an Haupthistokompatibilitätskomplex (major histocompatibility complex, MHC) Klasse I oder II gebundenes Antigen an naive T Zellen zu präsentieren. DCs besitzen die einzigartige Funktion nach einem zusätzlichen Aktivierungsstimulus exogenes Antigen an CTLs kreuz-präsentieren zu können. Dieses Signal, das durch die Bindung an von aktivierten Helfer-T Zellen exprimiertes CD40L an DCs vermittelt wird, ist als „Lizensierungs-Signal" bekannt. Die Aktivierung von Tumorantigen-spezifischen CTLs ist ein zentraler angestrebter Schritt in der anti-Tumor Vakzinierung.

In vitro differenzierte DCs, die weiters mit Lipopolysaccharid (LPS) als Toll-like Rezeptor (TLR)-4 Ligand ausgereift wurden, zeigen einen dynamischen Phänotyp der kurzzeitig nach der Reifung immun-stimulatorisch, später jedoch immun-regulatorisch oder suppressiv wirkt. Als Indikator dient dabei die Expression des Helfer T Zell Typ 1 (Th1) polarisierenden Interleukins (IL)-12. Dieses wird während der immun-stimulatorischen Phase von DCs sekretiert, wohingegen später die Expression von IL-10 regulatorisch auf eine Immunreaktionen wirkt. Wir untersuchten den Effekt von ektopisch exprimierten CD40L auf DCs, das durch einen lentiviralen Gentransfer in 6 Stunden (reifende DCS, semi-mature DCs, smDCs) oder 48 Stunden LPS/IFN-γ vor-gereifte DCs (reife DCs, fully mature DCs, mDC) eingebracht wurde. Reife DCs, deren IL-12 Expression bereits erschöpft ist konnten mittels CD40L, aber nicht mittels GFP Gentransfer zur IL-12 Expression re-stimuliert werden. In einer Ko-Kultur von CD40L exprimierenden mDCs mit T Zellen eines anderen Spenders konnte beobachtet werden, dass vermehrt CTLs mit einer verstärkten lytischen Kapazität, gemessen durch Granzyme-B (GrB) Expression, expandiert sind. Daraus schließen wir, dass das

durch CD40L vermittelte Signal an DCs den immune-stimulatorischen Phänotyp verstärkt. Diese Beobachtungen haben einen starken Einfluss auf das zukünftige Design von DC-basierender anti-Tumor-Vakzinierung.

Abbreviations

alloMLR	Allogeneic mixed lymphocyte reaction
APC	Allophycocyanin
APCs	Antigen presenting cells
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CTL	Cytotoxic T lymphocyte
DAPI	4',6-Diamidino-2-phenylindole
DC	Dendritic cell
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescent activated cell sorter
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box protein 3
GFP	Green fluorescent protein
GM-CSF	Granulocyte monocyte colony stimulating factor
IFN	Interferon
iDC	Immature DC
IL	Interleukin
LC	Langerhans cell
LPS	Lipopolysaccharide
mDC	Mature DC
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
pDC	Plasmacytoid DC
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PI	Propidium iodid
preDC	Precursor of DC
smDC	Semi-mature DC
TAP	Transporter associated with antigen presentation
Th	T helper cell
Th1/Th2	T helper cell type 1/2
TLR	Toll like receptor
TNF	Tumor necrosis factor
7-AAD	7-Amino-actinomycin

1 Introduction

1.1 Dendritic cells and the immune system

Multicellular organisms evolved diverse defence mechanisms against pathogenic invaders. As soon as infection is detected the innate immune system becomes activated. Innate immunity acts in a non-specific manner and represents the first barrier for hostile pathogens in the body. Its responses to pathogens reduce infection and activate components of adaptive immunity. In detail, it activates antigen-presenting cells (APCs) that are needed to trigger adaptive immunity and increase the specificity of the immune reaction to further on generate memory [5].

Dendritic cells (DC) as the main APCs in the body play a pivotal role in the stimulation of the immune system [6]. Depending on maturation status DCs can act tolerogenic or as main stimulators of immune reactions. Immature DCs (iDCs) reside at pathogen-entry sites surveying the surroundings for potential danger for the body like inflammation or tissue damage and continuously sample the antigenic environment [2]. At this activation state iDCs induce tolerance against self antigens.

To detect bacterial or viral infection DCs express toll like receptors (TLRs) that recognise pathogen associated molecular patterns (PAMPs). These PAMPs are small molecular motives that can be found in bacterial or viral surface proteins, which were termed by Matzinger and colleagues danger signals [7]. Upon maturation by TLR mediated signals indicating infections [8] or injured self phenotypical changes arise in the DC, like changes in the expression of adhesion molecules, homing receptors, strong up-regulation of T-cell co-stimulatory molecules CD80 and CD86 and increased expression of processed antigen bound to major histocompatibility complex (MHC). During migration to the lymph node these phenotypic changes, which include expression of the T helper type 1 (Th1) polarizing cytokine interleukin (IL)-12 [9], develop. After this early phase of activation the DC again shifts its phenotype, indicated by exhaustion of the ability to secret Interleukin (IL)-12 [10] and immunosuppressive feedback mechanisms start to outweigh the immune stimulatory potential of DCs (Figure 1).

DCs were first identified by the German anatomist Paul Langerhans in 1868. He discovered one DC subset located in the skin, called Langerhans cells (LC). Unfortunately, he mistook them for nerve endings [6]. Nearly 100 years later DCs were rediscovered by Ralph M. Steinman and colleagues, who purified them from murine lymphoid organs and realized that DCs are part of the immune system [1]. Since then DCs were intensively studied for their key function as mediators of immune reactions.

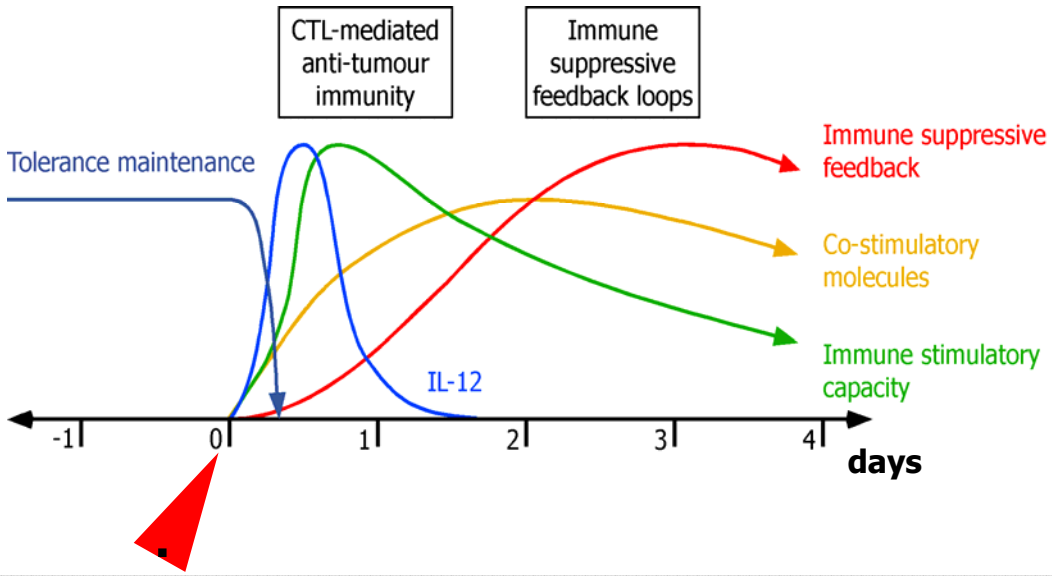

Figure 1|Plasticity of the phenotype of DCs: Tolerogenic phenotype is shown until DCs encounter maturation stimulus (indicated by red arrow). During the first phase of maturation, DCs start to up-regulate co-stimulatory molecules. Confering high immune stimulatory capacity the DCs express Interleukin (IL)-12, but after 48 hours DCs have exhausted their capacity for IL-12 secretion. In our model, immune suppressive mechanisms start to become up-regulated by the DC after an early phase of immune stimulation indicated by IL-12 expression.

1.2 Differentiation of DCs from precursor cells

1.2.1 General information

DCs are comprised of subsets that differ from one another in terms of location, antigen presentation and maturation [11-13]. All DC subsets arise from precursor cells that are located in the bone marrow [14]. DCs are found circulating in the blood and take up residence in fully differentiated but immature form in organs like skin, spleen, lung or mucous membranes [6]. In elevated numbers DCs can be found at

sites of inflammation [15]. As DCs are known to have several roles in the immune system like interacting with T cells, B cells and natural killer (NK) cells, all these functions can not be carried out by one single cell type. Therefore different subsets of DCs are expected to have different functions [2]. There are two models that might explain the appearance of different DC subsets in the murine and the human system. One is that different functions reflect diverse differentiation stages of one DC lineage. The idea behind this model is that the functional differences are entirely depending on local environmental signals [2]. The other model suggests that specialized DC subtypes represents differentiated DCs from different cell lineages [2]. It is assumed that in reality both models exists and interfere to some extent [2]. There are many subtypes of DCs described in the murine system. However, this introduction will focus on human DCs only.

1.2.2 Human DCs subtypes

In contrast to the murine system where matured DCs can be isolated *in vitro* out of spleenic tissue, in the human system mainly immature DCs (iDCs) or precursors of DCs (preDCs) can be found in the blood. These precursors of DCs are called monocytes. *In vitro* the monocytes may be differentiated into DCs with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 [16].

Generally spoken two different DC subsets have been identified yet: myeloid DCs and plasmacytoid DCs (pDCs). pDCs, but not myeloid DCs, express toll like receptor (TLR) 7 and 9 and therefore can detect single stranded (ss) RNA and DNA viruses [17, 18]. Further on precursors of pDCs lack T cell receptor alpha (TCRα), TCRβ , TCRγ , TCRδ and CD3 chain but express CD4 surface molecules [19]. After maturation by viral or bacterial stimuli pDCs express large amounts of alpha/beta interferons (IFN-α/β) and thus are identified as "natural type I IFN-producing cells" in the blood [19-21]. These two DC subsets arise from haematopoietic precursor cells in the bone marrow through yet not fully characterized intermediates to more differentiated precursor cells (preDC) circulating in the blood until these cells up-regulate homing receptors to peripheral non-lymphoid tissues where they reside as immature DCs (Figure 2) [18].

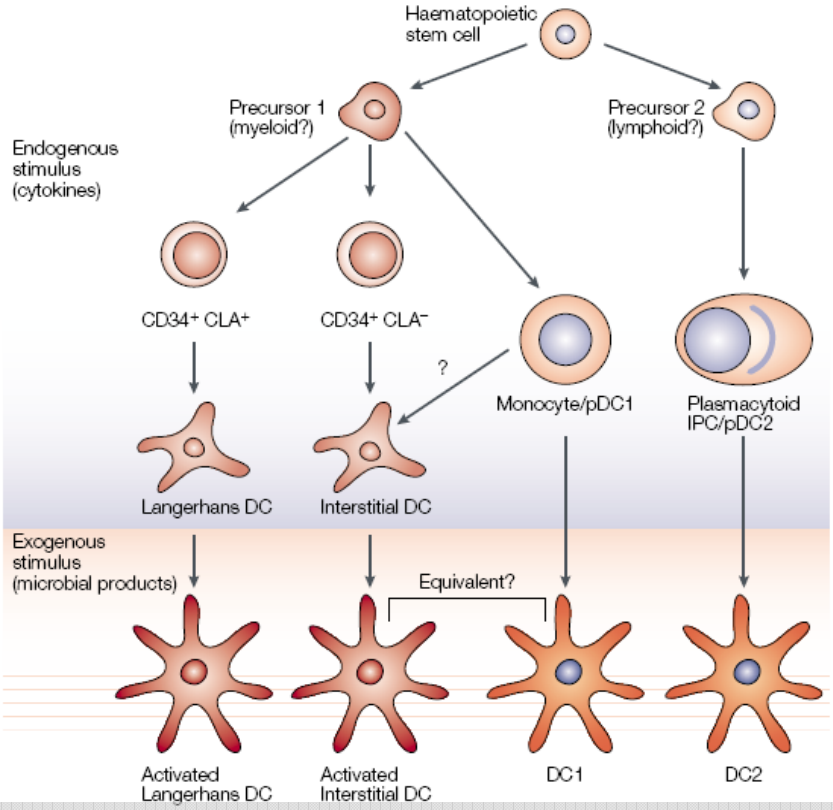

Figure 2|Pathways of DC differentiation: DCs arise from haematopoietic stem cells by two lineages: lymphoid or myeloid. Endogenous stimuli like cytokines can generate DCs that are matured but need further exogenous stimuli to be fully activated. On the other hand cytokines also can lead to differentiation but maturation is achieved only by further activation with exogenous stimuli like microbial products. (pDC1=precursor of DC1) Picture was taken from reference # [2].

Though human and murine DCs cannot be compared because they lack the same kind of surface molecules (like CD8 as a typical murine DC surface molecule) one major DC subset can be found in both individuals as offspring of bone marrow precursor cells by differentiation through the myeloid pathway: the langerhans cells (LC) that typically express langerin [2]. LCs as typical APCs are located in the skin where they remain in an immature state until they get activated. After activation they leave the periphery of the body and travel to the lymph node where they stimulate naive T cells with processed antigen presented on MHC molecules. Intense studies on LC caused the DC-migration-paradigm [22], which postulates that after migration DCs start to leave the periphery and travel to the draining lymph nodes. In contrary, recent studies showed that also iDCs are found in secondary lymphoid tissues. Therefore migration capacity does not automatically mean maturation [22]. Huang

and colleagues observed that DCs can be found in lymph nodes bearing fragments of apoptotic cells [23]. *In vitro* studies showed that this DC sub-population stimulates allogeneic T cells in mixed lymphocyte reaction (MLR), where DCs and T cells from different donors are co-cultivated, but cannot stimulate activation of autologous T cells. These migrating iDCs promote tolerance instead [22-24].
In humans only 0,2 percent of the whole white blood cells are DCs and even less can be found in diverse tissues like the skin [6].

1.3 Maturation

1.3.1 Maturation of DCs in vivo

In the periphery of the body iDCs reside and sample antigen. At this stage iDCs have a high potential to capture and process antigen but do not provide any signals for T cell activation. In contrary, iDCs trigger tolerance to T lymphocytes in the periphery or in case of migrating iDCs, they stimulate T cell deletion or induce regulatory T cells. After maturation of the DC triggered by diverse maturation stimuli, the function of antigen presentation is up-regulated, detectable by increased amounts of MHC class I and II molecules expressed on the cell surface. In parallel, T cell co-stimulatory molecules (CD80, CD86) and CD40 are up-regulated and expressed on the cell surface. Including IL-12 cytokine secretion, the DC now possesses a whole repertoire to prime T cell responses (Figure 6).

Generally, DCs have diverse receptors up-regulated to survey their surroundings. One group of receptors that provides the DC with these sensory mechanisms are called toll like receptors (TLRs). There are 11 different TLRs known that are expressed on human cell types, from which 10 are expressed intracellular or on the cell surface of DCs [25]. TLRs recognise PAMPs that are structurally conserved molecules derived from microbes. TLR4 is expressed on cell surface of DCs and recognizes lipopolysaccharide (LPS), a component of bacterial cell wall. TLR3, TLR8 and TLR9 are expressed at the endosome or phagosome membrane and recognize double stranded RNA (dsRNA), single stranded RNA (ssRNA) or un-methylated CpG

DNA motives, respectively. Activation of TLRs in DCs leads to signal transduction by translocation of the nuclear factor NF-κB, followed by increased expression of co-stimulatory molecules and pro-inflammatory cytokines [25, 26].

DCs can also be matured by inflammatory cytokines like IFN-γ or by interaction of CD40L of activated helper T cells (Th) and CD40 of DCs (see also chapter 1.4 the licensing signal). In either case phenotypic changes arise in the DC like decreased antigen up-take and increased antigen presentation. Also co-stimulatory molecules CD80 and CD86 are up-regulated and the DC starts moving towards the lymph node. The DC reaches the lymph node at a matured stage and starts to activate antigen specific T cells.

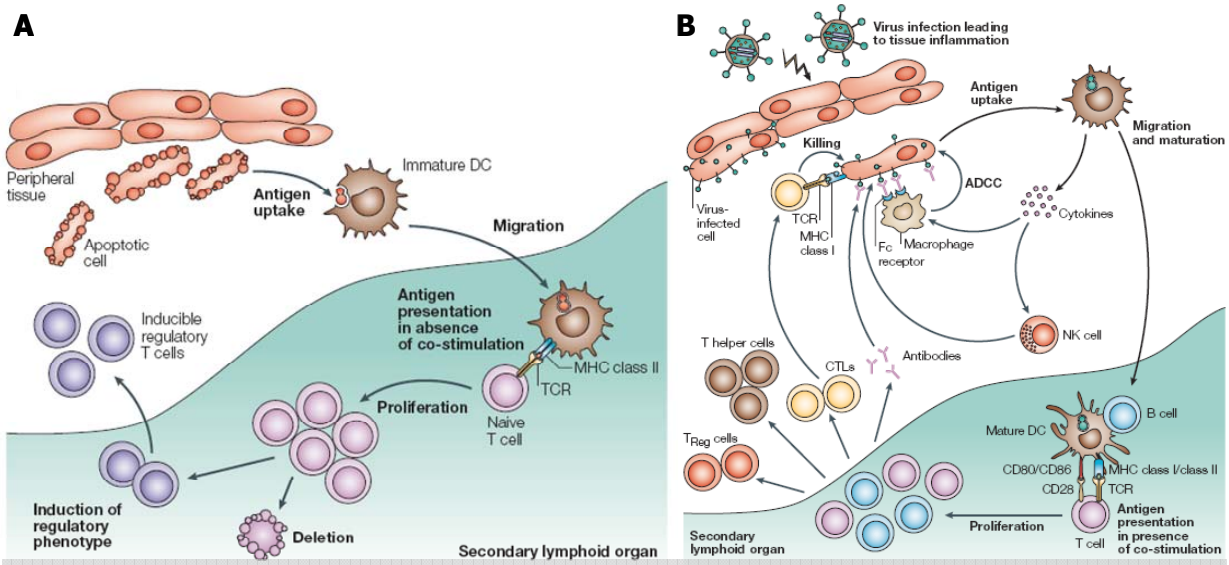

Figure 6|DCs have different functions depending on their activation status: This model from Banchereau and Palucka shows that immature DCs in the steady state can take up antigen from apoptotic cells and migrate without maturation into the lymph node (A). Without additional co-stimulatory signals the DC presents antigen to naïve T cells and leads to deletion of T cells or induces regulatory T cells. (B) Mature DCs show increased immunogenicity. In case of infection the DCs mature and migrate in large numbers to the lymph node. They present antigen-MHC complexes combined with co-stimulatory molecules to naive T cells and induce expansion of antigen-specific CTLs, T helper cells, B cells and also regulatory T cells to control the immune reaction. With the whole repertoire of adaptive immunity the infection can be eliminated.
ADCC=Antibody dependent cell-mediated cytotoxicity, also called opsonisation. CTL=cytotoxic T lymphocyte. T_{reg}=regulatory T cell. Picture is taken from reference #[3].

1.3.2 TLR4/IFN-γ matured DCs are used as anti-tumour vaccine

As DCs play a central role in controlling immunity they are a logical targets in many clinical situations that involve T cells [15]. There are different factors why DCs are used as anti-tumour vaccines: at first they activate and expand a whole repertoire of tumour-antigen specific effector cells (NK T cells, NK cells, αβ T cells and βδ T cells) and second *ex vivo* generated DCs retain their immunising capacities in cancer patients [13, 27]. Though there are many ways to trigger *ex vivo* generated DCs with maturation stimuli, I will only go into detail describing DCs used by our group [28]. We generate immature DCs (iDCs) from monocytes derived by elutriation, which are differentiated for 6 days with IL-4 and GM-CSF. These iDCs are then loaded with whole tumour cell lysate for 2 hours and afterwards are triggered by LPS/IFN-γ as maturation stimulus. Before maturation the DCs phagocytose the tumour proteins from the culture medium, process them and present them in MHC context on the cell surface. After 6 hours maturation (semi-matured DCs, smDCs), up-regulation of co-stimulatory molecules on the DC surface takes place. Now equipped with antigen presented on MHC (signal 1) and co-stimulatory molecules CD80 and CD86 (signal 2) for efficient T cell stimulation the DCs moreover expresses IL-12, which is triggered by LPS/IFN-γ maturation. Now 3 signals including expression of Th1 polarising cytokine IL-12 can be delivered to naïve T cells by injecting these smDC into the patient's lymph node. After completion of a phase I study using these kind of antigen-loaded smDCs [28] a phase II trail just recently started.

1.4 Licensing signal

As safety mechanism, cytotoxic T lymphocytes (CTLs) are generally inactive and need a further activation signal to develop killing capacity [29]. To trigger killing capacity, not only antigen bound to MHC class I is needed to be recognised by $CD8^+$ T cells but also additional help for activation from T helper (Th) cells is required. These Th cells have to be activated by the same antigen bound to MHC presented on an APC. Two models exist that suggest different applications of this licensing signal to $CD8^+$ T cells.

One model was first described by Keene and colleagues and postulates that Th cells and CTLs recognise the specific antigen simultaneously on the same APC [29, 30]. The rare event that one APC binds to antigen specific CTL and Th cell provides first evidence that there have to exist alternatives. The second model, also called "licensingl"-model, was first described in literature as an alternative to general knowledge based on theoretical background of Matzinger and colleagues [29, 31, 32]. In this model it is suggested that DCs as specialized professional APCs present exogenous antigen and are activated by a specific signal provided from Th cells. These fully activated "licensed" DCs then can stimulate CTLs [29, 33]. In detail, CD40L from activated Th cells and CD40, expressed on DC cell surface after maturation, are the two molecules responsible for this second activation signal on DCs [29, 34, 35]. This model also suggests that the rare event of APCs binding to antigen-specific T cells can occur sequentially not simultaneously.

1.5 Interaction of dendritic cells with T cells

1.5.1 Antigen presentation

In most tissues, DCs are present in an immature state. At this state they are unable to activate T cells because iDCs fail to have T cell co-stimulatory molecules and do not express stimulatory cytokines. Although iDCs lack these requisite accessory signals for T-cell activation they are extremely well equipped to capture antigens [15]. iDCs take up proteins by phagocytosis or by formation of large pinocytic vesicles (macropinocytosis) [9] and by receptor mediated endocytosis [36]. After antigen uptake, which also can provide signals for maturation, the DCs loose their ability to capture antigen but gain the ability to present antigen associated in major histocompatibility complex class I and II (MHC I and II) [15]. To be presented the antigen has to be processed into small peptide ligands for MHC molecules by the intracellular degradation machinery of the DCs.

There are two main ways of antigen degradation in DCs dependent on the source of antigen. Antigens can be characterized as endogenous, which means located in the

cytosol of the DC, or exogenous, that are products from other cells that are up-taken from the surroundings by the DC [1]. The peptides presented by MHC class I molecules are derived from proteins degraded mainly in the cytosol by the proteasome (Figure 3), whereas MHC class II molecules present peptides that are derived from proteins degraded in endosomal compartments by the cathepsins and other hydrolytic enzymes (Figure 4) [37].

1.5.1.1 Presentation of endogenous antigen on MHC class I molecules

All cells in the body have the ability to process defective ribosomal products or proteins by degradation using the proteasome. After degradation the processed antigen is transported into the lumen of the endoplasmatic reticulum (ER) using the transporter associated with antigen presentation (TAP). In the ER lumen freshly synthesised MHC class I molecules are retained. The imported processed antigens are then loaded on MHC class I molecules and expressed on the cell surface (Figure 3).

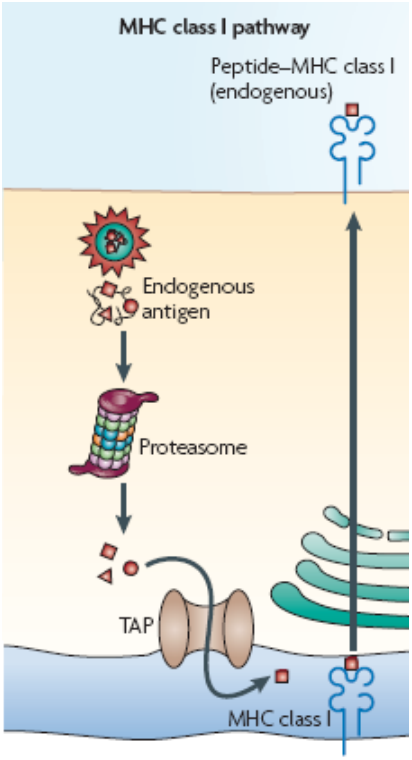

Figure 3 | Antigen presentation by MHC class I pathway of DC: Endogenous antigen like self antigen or viral proteins are processed by the proteasome and loaded on MHC class I in the endoplasmatic reticulum (ER, indicated as blue lumen). MHC class I molecules then are exported using the golgi apparatus (indicated as green) to be expressed on the cell surface. Picture taken from reference # [1]

In case of viral infection the virus uses the protein production machinery of the host to synthesise all viral specific proteins that are needed for replication. These proteins also are degraded by the proteasome-degradation-pathway and are presented on MHC class I molecules. T cells that traffic the tissue looking after patterns that are not derived by the body itself can recognise these viral protein-MHC class I complexes. The T cell then kills the target cell to evade on-going infection and multiplication of viral particles.

1.5.1.2 Presentation of exogenous antigen on MHC class II molecules

In contrary to the MHC class I pathway exogenous antigens are presented on MHC class II molecule following another pathway of degradation [4]. In this pathway exogenous antigen or self-antigen that is processed by the endocytic pathway is degraded by different proteases, like cathepsins. Small Peptides are formed as intermediates during late endosomal protein degradation. The loading of MHC class II molecules during this endocytic route is triggered by the chaperone protein HLA-DM and antigen-MHC complexes are then transported to the cell surface (Figure 4) [4, 38]. Also self-antigens that are found in endosomal compartments of DCs are presented on MHC class II molecules [1]. This includes components like particles of the endocytic pathway, recycled membrane proteins and peptides derived from autophagy [1, 37, 39].

Antigen loaded on MHC class I or class II can be recognised by the T cell receptor (TCR) of naïve T cells in the lymph node, in detail $CD4^+$ helper T cells bind antigen loaded on MHC class II molecules, whereas $CD8^+$ CTLs only detect antigen-MHC class I complexes. Recognition alone can not activate T cells but further binding of co-stimulatory molecules from DCs to CD28 molecules expressed on the surface of T cell is needed. During maturation the DCs start to migrate into the lymph node and up-regulate not only processed antigen in MHC context but also CD80 and CD86 for T cell co-stimulation (see also chapter 1.6. Effector functions of DCs).

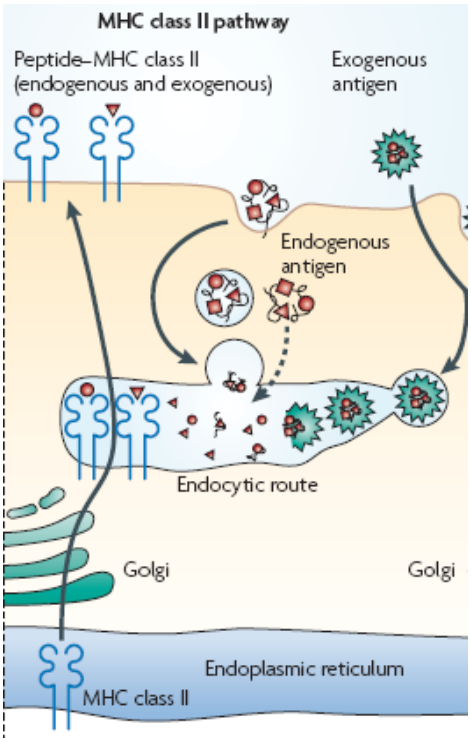

Figure 4 | MHC class II pathway of antigen presentation by DC Exogenous antigen is uptaken and processed by the endocytic degradation route. Peptides are then loaded on MHC class II molecules and presented on cell surface. Picture taken from reference # [1]

1.5.2 Cross presentation

To stimulate $CD8^+$ T cells against antigens that are not expressed by the DC (e.g. tumour antigens or antigens derived from viruses that do

not infect DCs), exogenous antigen has to be presented by DCs in MHC class I context. This antigen presentation pathway differs from the classical presentation of exogenous antigens, which are normally bound to MHC class II molecules [4]. This process is called cross-presentation. As cross-presentation is likely to activate CTLs in response to vaccine antigens it is a intensively studied mechanism but not very well understood by now. Different suggestions exist for this switch of MHC molecule class (Figure 5).

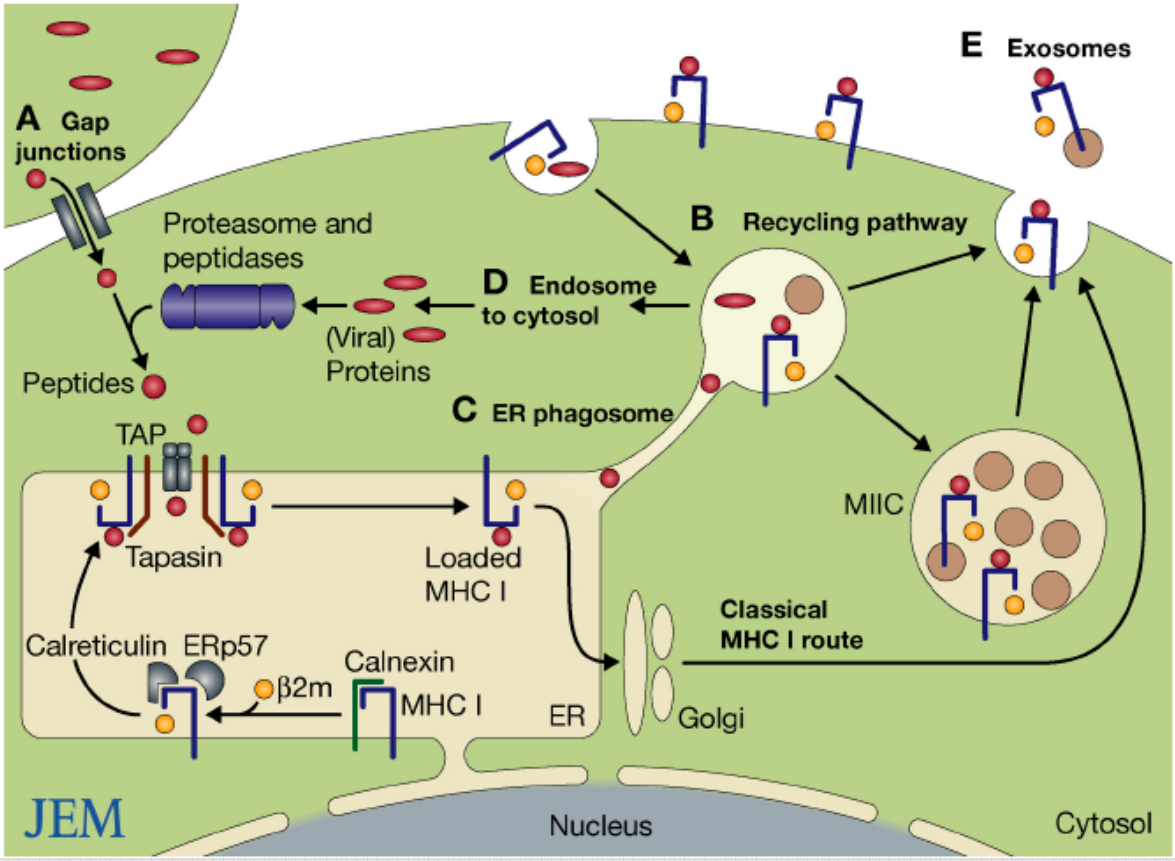

Figure 5|Different models of cross-presentation: Classical presentation of intracellular antigens is restricted to MHC class I molecules. Intracellular antigen are processed by the proteasome or peptidases and transferred into the endoplasmatic reticulum (ER) using the transporter associated with antigen presentation (TAP). In the lumen of the ER newly synthesized MHC class I molecules are loaded with the antigen and presented on the cell surface. For cross-presentation exogenous antigens enter this classical MHC class I route in different ways.
(A) Peptides from neighbouring cells can be directly transferred to the cytosol of DCs using gap junctions.
(B) Also recycling of MHC class I molecules can lead to re-load by exogenous antigen in endosomes.
(C) Fusion of compartments from the phagosomal route and the ER lead to transfer of uptaken exogenous antigen into the lumen of the ER.
(D) Antigens can also leave the endosome using export mechanisms into the cytosol.
(E) Exosomal derived antigens can bind to DC surface and be up-taken.
Picture is taken from reference # [4]

Neiijssen and colleagues showed that intracellular peptides can be transferred from one cell to neighbouring monocytes by using gap junctions (Figure 5A) [40]. Studies also showed that extracellular proteins enter the cytosol of DCs and make them available for proteasomal degradation. This is then followed by transport to the ER using transporter associated with antigen presentation (TAP) and presentation on the cell surface after loading to MHC class I molecules in the lumen of the ER (Figure 5E, C, D) [41-43]. Gromme and colleagues observed that MHC class I molecules can be recycled from the cell surface and may load exogenous antigen during recycling by the endocytic pathway (Figure 5B) [4, 44].

1.6 Effector functions of DCs

Mature DCs are defined by their ability to trigger T cell stimulation by delivering three different signals [22]. Directly, these three signals define the specific T cell effector function that is polarized after activation by DCs [45].

1.6.1 Signal 1 - Antigen presentation

Signal 1 is derived from TCR binding to antigen-MHC complex presented by DCs. This signal alone cannot induce T cell stimulation. Without further activation signals the DCs inactivate naive T cells and induce tolerance. (As described in chapter 1.5.1 antigen presentation)

1.6.2 Signal 2 - Co-stimulation

Signal 2 is the additional signal triggered by co-stimulation of T cells by the DC that, together with signal 1, induces immunity. Co-stimulation is performed by binding of CD28 on T cell surface to CD80/CD86 expressed by DCs [22, 46].

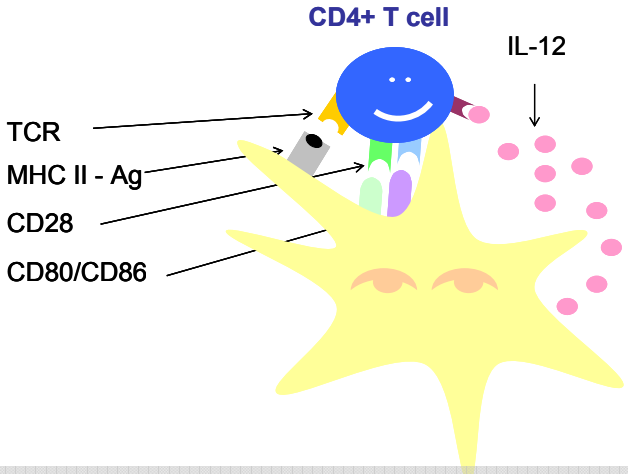

Figure 6|DC activates T helper cell: The matured DC stimulates T helper cells by application of 3 signals: At first, antigen presentation (interaction of TCR and MHC), second co-stimulation with CD80/CD86 binding to CD28 and as signal 3 it polarises T cell development by secretion of polarising cytokines.

Signal 2 makes the decision if tolerance or an immune response takes place, as not only T cell stimulatory molecules like CD28 can be bound but also cytotoxic T-lymphocyte antigen 4 (CTLA4) shows high affinity to CD80/CD86, that further on leads to tolerogenic or suppressive signals [22, 47, 48]. CTLA4 is only expressed on activated T cells [49] and signal transduction by CTLA4 stimulates suppressive effects.

1.6.3 Signal 3 – The polarisation signal

Signal 3 was recently added to the terminology and describes signals from DCs that trigger the differentiation of stimulated T cells to effector cells [22, 50]. After antigen presentation on MHC complexes to TCR, co-stimulatory signals by CD80/CD86 binding, an additional cytokine-secretion profile as signal 3 is important to raise antigen specific effector functions in T cells. These cytokines mediate immunity against pathogens infecting the host.

Th1 cells secrete IFN-γ, and tumour necrosis factor-α to raise cellular immunity against intracellular bacteria or viral infection [51].
Th2 cells activate humoral immunity against extracellular parasites by producing Interleukin (IL)-4, IL-5 and IL-13 [51]. The DC delivers again one critical signal to prime immune responses by activating T cell effector functions as it expresses the Th1 polarising cytokine IL-12 [52]. Recent data suggests that INF-γ, provided by a third cell (presumably NK cells, or γδ T cells), beside IL-12 is critical for Th1 polarisation [53]. On the contrary, without IL-12 expressed from DCs mast cells, NK T cells, basophils, eosinophiles and again γδ T cells produce IL-4 and support Th2 polarisation [51].

Together these 3 signals provided from matured DCs give rise to multiple types of T cell differentiation including immunity, tolerance and immune evasion [22].

1.7 Working hypothesis

In *in vitro* experiments one always tries to model the *in vivo* situation. In our DC model we mature DCs by administration of lipopolysacharide (LPS) and interferon gamma (IFN-γ) to resemble a bacterial infection (Figure 1). During bacterial infection TLR4 is stimulated *in vivo* by LPS and additionally IFN-γ is supplemented by the activated innate immunity as key pro-inflammatory cytokine. Early after maturation, when IL-12 is highly expressed by DCs, the DC is called semi-mature (smDC). After some time DCs get fully matured (mDCs) and highly up-regulate phenotypic maturation molecules but IL-12 expression is terminated. At this maturation stage DCs are called "exhausted" for their ability to secrete IL-12. In the *in vivo* situation, maturing DCs reach the lymph node and stimulate Th cell proliferation by interaction of antigen loaded MHC II (exogenous antigen) with the TCR/CD3 complex under additional co-stimulation by the B.7 family molecule members (e.g. CD80 and CD86). CD40 that is up-regulated on the surface of mature DCs then interacts with CD40L of Th cell and triggers cross-presentation. After this licensing signal the DC is able to express exogenous antigen in MHC class I context, a mechanism that is still not fully clarified. Yet the DC can present antigens derived from viruses or tumour by phagocytosis to $CD8^+$ T cells and activate them by co-stimulation.

We postulated the following working hypothesis: By ligation of ectopically expressed CD40L on DCs to CD40 (Figure 7) we can administer an additional maturation signal to fully matured IL-12 exhausted DCs. These "exhausted" DCs can then be re-activated by a second wave of IL-12 expression triggered by CD40-CD40L ligation. Up-coming negative regulatory feedback mechanisms are diminished by the immune stimulatory capacity of the re-activated DC. Exogenous antigen then can be presented to CTLs and further on Th1 polarised T cell stimulation is induced (Figure 7). After activation and expansion of antigen specific CTLs the infection in the periphery can be eliminated.

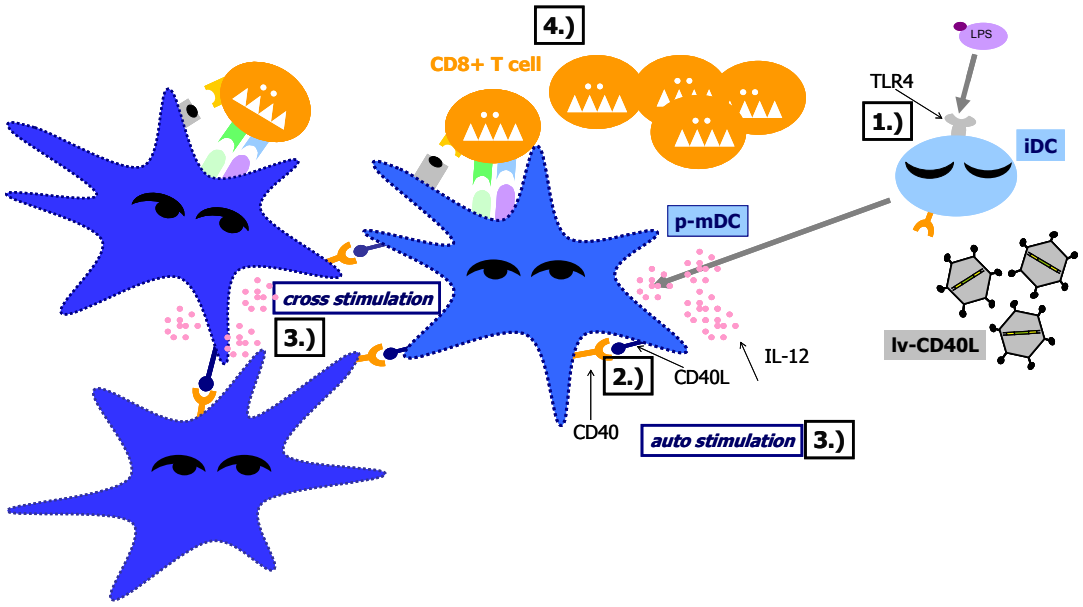

Fig 7 | CD40L gene transfer into pre-matured DCs by lentiviral transduction: iDCs are matured by TLR4 activation with LPS (1) and immediately afterwards transduced with lentiviral particles containing genetic information for CD40L expression. In co-culture with T cells, the CD40L expressing DC (2) can now stimulate itself by CD40-CD40L interaction or cross stimulate other DCs (3). CD40-CD40L interaction delivers the *licensing* signal to DCs which then present exogenous antigen in MHC I context and activate $CD8^+$ T cells.

This model implies that fully matured DCs are able to receive a second activation stimulus which extends their capacity to act as immune stimulators. In our model, this immune stimulation is IL-12 dependent and can be re-induced in DCs with exhausted capacity for IL-12 expression by CD40-CD40L ligation. This concept is controversial as most investigators consider DCs terminally differentiated with exhausted capacity for IL-12 expression after one single maturation stimulus and the resistance to any further maturation signal.

1.8 Specific aims

The aim of my project was to investigate the function of CD40L ectopically expressed on DCs to further understand the impact of CD40L as a critical part in our model of two step maturation. This model suggests that DCs at peripheral infection sites are matured by PAMP engagement and pro-inflammatory cytokines mimicked in our *in vitro* DC maturation strategy by LPS and IFN-γ supplementation. After migration into the lymph node the DCs receive a second CD40-CD40L derived signal ligation and thus develop into professional APCs with the ability to cross-present exogenous

antigen and prime CTLs. CD40L was directly expressed on LPS/IFN-γ activated DCs in order to investigate the cytopathic effects of lentiviral transfer. The effect of CD40L expressed on DCs at different maturation stages (smDCs and mDCs, as described in chapter 1.3.2. TLR4/IFN-γ matured DCs are used as anti-tumour vaccine) was determined by measuring phenotypic alterations of the DCs including cytokine secretion and T cell stimulatory potential in an allogeneic setting.

2 Material and Methods

2.1 Primary Cells and Cell Lines

2.1.1 Dendritic cells

Leukocytes were collected using an Amicus leukocyte apheresis device (Baxter, Deerfield, IL) from healthy volunteers. For the enrichment of monocytes from PBMCs in the leukocyte apheresis product the Elutra cell separator (Gambro BCT, Lakewood, CO) was used by following the instructions of the manufacturer. Basically, the cells are separated by sedimentation velocity, dependening on cell-size and density. After separation the cells were analysed by flow cytometry, by labelling the cells with monoclonal antibodies directed against CD3, CD4, CD8, CD19, CD14, and CD15 (antibodies from BD Pharmingen, San Diego, CA) in order to detect total T-lymphocytes, T-helper cells, cytotoxic T-lymphocytes, B-lymphocytes, monocytes, and granulocytes, respectively. Labelled cells were analysed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) and data analysis was performed using CellQuest software (Becton Dickinson, Mountain View, CA). If not stated otherwise, the same data analysis software and FACS flow cytometer was used in all further settings.

2.1.1.1 Differentiation

Monocytes, enriched from the isolation process by elutration described above were differentiated by following a previously optimised protocol for generating DCs [54]. In detail, monocytes were cultured at a starting density of $1x10^6$ monocytes/cm^2 in CellGro medium supplemented with 1000 U/ml recombinant human GM-CSF and 300 U/ml recombinant human IL-4 (all from CellGenix, Freiburg, Germany) at 37°C and 5 % CO_2 for 6 days. On day 3 the cells were fed with the same volume of CellGro plus GM-CSF and IL-4.

2.1.1.2 Maturation

Maturation was carried out on day 5 or day 7, depending on experimental setup, by adding 50 ng/ml IFN-γ (Boehringer Ingelheim, Vienna, Austria) and 1000 ng/ml lipopolysaccharide (LPS, *E. coli* strain O111:B4, Calbiochem, San Diego, CA, USA) to the culture for 6 hours to generate semi-mature (sm) DCs, or continuously for 48 hours to generate fully-mature (m) DCs. In case of lentiviral transduction the cells were washed after 6 hours or 48 hours and transduces with lentiviral supernatant in presence of 50 ng/ml IFN-γ and 6 µg/ml hexadimethrine bromide (Polybrene; Sigma-Aldrich, St. Louis, MO) for another 6 hours. For detailed information see also chapter 2.4.2 Lentiviral transduction of DCs.

2.1.2 Peripheral Blood Mononuclear Cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) were isolated from Buffy Coat, obtained from healthy blood donors at the blood bank of General Hospital Vienna by centrifugation over Ficoll Paque™ PLUS density gradient (Amersham Biosciences, Uppsala, Sweden). PBMCs were collected from interphase after centrifugation at 665xg at 20°C for 20 minutes and washed twice with 1x D-PBS (Invitrogen, Grand Island, NY), total cell number was assessed by SYSMEX KX-21 CBC analyzer (Sysmex Deutschland GmbH, Norderstedt, Germany) and cells were resuspended in AIM-V medium (Invitrogen Corporation, Bethesda, MD) with 2% pooled human AB plasma (Octaplas, OP, Octapharm, Vienna, Austria)(AIM-V/OP). Cells were frozen at -80°C in portions of $5x10^7$ cells in freezing medium, consisting of 40% AIM-V/OP, 40% OP and 20% DMSO (Dimethylsulfoxid, SERVA, Heidelberg, Germany). After freezing the cells were stored in liquid nitrogen until further use.

2.1.2.1 Purification of $CD3^+$ T cells

To have a detailed look at the proliferation potential of $CD4^+$ and $CD8^+$ T cells in co-culture with CD40L expressing DCs (CD40L-lv-DC), $CD3^+$ T cells were isolated from PBMCs by cell depletion based on magnetic bead cell sorting using a pan T cell

isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMCs isolated from Ficoll density gradient centrifugation were thawed at 37°C water bath and immediately diluted in AIM-V/OP for further washing steps. Standard centrifugation velocity was 483xg at 4°C for 7 minutes. If not stated otherwise, these settings were used for all cell types. Isolation of $CD3^+$ T cells by negative depletions means indirect labelling of all $CD3^-$ non T cells with biotin-conjugated antibodies directed against CD14 (macrophage marker), CD16 (NK cells), CD19 (B cell marker), CD36 (platelet marker), CD56 (NK cells), CD123 (plasmacytoid dendritic cell and basophilic granulocyte marker) and CD235a (Glycophorin A, erythroide cell marker). These antibodies then bind to anti-biotin magnetic beads which are retained in a column placed into a magnetic field. After two washing steps all untouched CD3 purified T cells were flushed through the column and collected in flow-through. Aliquots before and after depletion were compared to calculate purity of $CD3^+$ T cells.

2.1.2.2 Proliferation assay using CFSE labelling of CD3 purified T cells

After two washing steps with 1x D-PBS the CD3 purified T cells were resuspended in 1x D-PBS, 0,1 %BSA at concentration of $1x10^7$ cells/ml and labelled with 5 μM Carboxy-fluorescein-succinimidyl ester (CFSE; Invitrogen Corporation, Bethesda, MD) for 10 min at 37°C with slight shaking every 2 min. Cells were then re-covered by addition of 10 times the volume OP to D-PBS,01 %BSA solution and incubated for 5 min at RT. CD3 purified CFSE labelled cells were then washed using AIM-V, 2%OP and number of lymphocytes was measured using SYSMEX KX-21 CBC analyzer (Sysmex Deutschland GmbH, Norderstedt, Germany) if not stated otherwise. Proliferation was measured by CFSE-dilution in context of an allogeneic mixed lymphocyte reaction (alloMLR, see chapter 2.1.2.3 Allogeneic Mixed Lymphocyte Reaction).

2.1.2.3 Allogeneic Mixed Lymphocyte Reaction (alloMLR)

$CD3^+$ CFSE labelled T cells were co-cultivated in total volume of 200 µl AIM-V/OP in a 96 well round bottom plate at density of 10^5 cells/100µl/well together with

stimulating allogeneic DCs at a 1:3, 1:9 and 1:27 DC : T cell ratio. The DCs were TLR4 pre-matured for either 6 hours (smDC) or 48 hours (mDC) with LPS/IFN-γ at concentrations described above and both groups were transduced with lentiviral particles, containing genetic information for CD40L or GFP (lvCD40l or lvGFP, respectively), or left untreated (nolv; see also chapter 2.4.2 Lentiviral transduction of DCs). As control for the proliferation capacity of $CD3^+$ cells the T cells were co-cultivated with cocktail mixture of staphylococcal exotoxins A and B (SEA/SEB; Toxin Technology Incorporation, Sarasota, FL) at concentrations of 200 ng/ml. To show the background proliferation potential of un-stimulated $CD3^+$ cells the T cells were incubated with AIM-V/OP only. On day 6 50 µl of supernatant was collected and frozen at -20°C for cytokine analysis by Flow Cytometric Bead Array (Bender Med Systems, see below) and cells were harvested and fixed with Cytofix/Cytoperm (BD Pharmigen). The proliferation and activity potential of $CD4^+$ and $CD8^+$ T cells was measured by CFSE dilution in dividing cells and CD25 positivity. For analysis of cytolytic activity of $CD8^+$ T cells intracellular granzyme B (GrB) expression was measured. GrB is the key component of the cytotoxic lymphocyte granules that can trigger an apoptotic caspase cascade in target cells [55]. Cells were then analysed for proliferation determining absolute number or percentage of CFSE negative cells (see chapter 2.2. Flow cytometry).

2.1.3 HeLa

The HeLa cell line is derived from an aggressive glandular cervical cancer [56]. After thawing the cells at 37°C, the cells were kept in 1650 RPMI (Euroclone, Milan, Italy) supplemented with 10 % FCS (PAA, Pasching, Austria) and split at stages of 80 - 100 % confluence. The cells were detached by applying 1/10 of the original culture-volume Accutase (PAA, Pasching, Austria) for 15 minutes.

2.1.4 293FT

The 293FT cell line (Invitrogen Corporation, Bethesda, MD) is derived from the 293F cell line [57] and stably expresses the SV40 large T antigen from the pCMVSPORT6TAg.neo plasmid. Expression of the SV40 large T antigen is controlled

by the human cytomegalovirus (CMV) promoter and is high-level and constitutive. Usage of 293FT Cell line allows generation of lentiviral constructs by co-transfection of plasmids containing genetic information of packaging and reverse transcriptase function (see chapter 2.4.1 Production of lentiviral particles).
293FT cells were cultivated in DMEM (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10 % FCS, 0,1mM MEM Non-Essential Amino Acids, 2mM L-glutamine (PAA, Pasching, Austria) and 500 µg/ml geneticin (Invitrogen Corporation, Bethesda, MD) and passed when they were over 80 % confluent after incubation with Accutase.

2.2 Flow Cytometry

To measure the maturation status of DCs, GFP or CD40L expression after lentiviral gene transfer and proliferation of T cell subsets in alloMLR, cells were analysed using FACS flow cytometry. 10^5 cells were harvested and FC receptors were blocked with µg human purified Ig (Sandoglobulin) for at least 15 min at 4°C to reduce unspecific immunofluorescent staining. Surface antigens of interest were then detected with antigen specific fluorochrome-conjugated monoclonal antibodies for 30 min at 4°C.

2.2.1 Surface staining

Phenotype of DCs after maturation was determined by measuring surface molecule densities CD80, CD86, CD83, MHC I and MHC II (Table 1). To determine purity of CD3 depleted lymphocytes cells were analysed with anti-CD45, anti-CD14, anti-CD3 and anti-CD56 antibodies to detect amount of un-depleted cells.

2.2.2 Intracellular staining

Proliferation of T-cells after alloMLR were analysed for surface expression of CD3, CD4, CD8, and CD25. Intracellular molecules like Granzyme-B or FoxP3 transcription factor were detected by intracellular staining using Cytofix/Cytoperm fixation kit (BD Pharmingen, San Diego, CA). After staining of cells surface antigens cells were fixed by application of 250 µl Fixation/Permeabilization solution for 20 min at 4°C. Two

washing steps with 1x BD Perm/Wash solution were performed prior to application of anti-Granzyme B (all from BD Pharmingen, San Diego, CA) and anti-FoxP3 (eBioscience) antibodies. After final washing step with 1xPBS 3-5x10^4 cells were acquired for determining total cell number with Trucount system techniques or percentage of CFSE negative cells using FACS Calibur or FACS LSRII flow cytometer and data analysis was performed with FACS CellQuest or FACS DIVA software (all purchased from Becton Dickinson, Mountain View, CA). Viability of cells was measured with propidium iodide (Pi), 7-aminoactinomycin (7-AAD) or 4',6-diamidino-2-phenylindole (DAPI). All appropriate isotype control antibodies were included in analysis.

Label	Fluor chrome	Company	Clone
anti-CD3	PE-TR/APC	BD Pharmingen	/SK7
anti-CD4	PerCP	BD Pharmingen	SK3
anti-CD8	APC-Cy7	BD Pharmingen	SK-1
anti-CD14	FITC	DAKo	TÜK4
anti-CD25	Alexa Fluor 647	Serotec	MEM-181
anti-CD40L	PE	BD Pharmingen	TRAP1
anti-CD45	PerCP	BD Pharmingen	Hle-1
anti- CD56	PE	BD Pharmingen	NCAM 16.2
anti-CD80	PE	Immunotech	MAB104
anti-CD83	APC	BD Pharmingen	HB15e
anti-CD86	FITC	BD Pharmingen	2331
anti-MHC I	PE	DAKO	W6/32
anti-MHC II	FITC	DAKO	CR3/43
anti-granzymeB	Alexa Fluor 700	BD Pharmingen	GB11
anti-Vδ2	PE	BD Pharmingen	B6
anti-FoxP3	APC	eBioscience	PCH101

Table 1|Antibodies used for flow cytometry

2.3 Cytokine Detection

2.3.1 Enzyme linked immunosorbent assay (ELISA)

Supernatant of DC cultures were analyzed 48 hours after maturation with LPS/IFN-γ at standard concentrations (see DC maturation) or lentiviral transduction with lvCD40L or lvGFP for IL-12 and IL-10 expressions using conventional Enzyme linked immunosorbent assay (ELISA) two-sandwich technique.
One 96-well plate (Nunc Maxisorp, Roskilde, Denmark) was coated overnight at 4°C with appropriate capture antibody diluted to final concentration in 1x D-PBS with 0,02% sodium azide (Merk, Darmstadt, Germany). The other day the plate was washed using ELISA-Washers (Skan Washer 400, SCATRON), followed by administration of 250 µl 2% bovine serum albumin (BSA; Sigma-Aldrich, St.Louis, MO) diluted in 1x D-PBS per well for 3 h for blocking reaction, evading unspecific binding to Fc receptors (FcR). After blocking, standard solutions of known concentration ranging from 62,5 ng/ml to 4000 ng/ml IL-10 or IL-12 protein and diluted samples were distributed in duplicates into the wells and incubated over night at RT.

cytokine	capture-AB	conc	detection-AB	conc
IL-12	anti-human IL-12p70	2 µg/ml	Biotin anti-human IL-12p40/p70	50 ng/ml
IL-10	anti-human IL-10	0,5 µg/ml	Anti-human IL-10	50 ng/ml

Table 2|Antibodies used for ELISA

The next day one washing step was followed by application of biotinylated detection antibody (Table 2; BD PharMingen, San Diego, CA) After incubation for 4 h at RT phosphatase conjugated streptavidin SA110 (Chemicon International, CA) was added in concentrations of 1:1000 in 1xD-PBS/1%BSA per well for 1h at RT. During this incubation step Sigma 104 DNPP phosphatase substrate tablets (Sigma Diagnostics, St. Louis, MO) were dissolved in diethanolamin buffer (1 tablet/5 ml buffer) and 100 µl were administered into every well. The plate was incubated in the dark until colour change was detected. The adsorption of the enzyme reaction was measured at 405-690 nm using a microtiter plate reader (Anthos Labtec Instruments, Salzburg,

Austria). Analysis was performed with Anthos WinRead software (Anthos Labtec Instruments, Salzburg, Austria).

2.4 CD40L gene transfer

2.4.1 Generation of lentiviral particles

Using ViraPower™ Lentiviral Expression System (Invitrogen, Carlsbad, CA) replication incompetent HIV-based lentiviral particles were generated. This gene transfer system can be used to deliver and express a gene of interest into non-dividing mammalian cells. As described in the manufacturer's manual the lentiviral cDNA is reverse transcribed once inside the cell and stably integrates into the genome using long terminal repeats (LTRs). Diverse modifications in the genetic material of the virus guarantees replication incompetence and high biosafety. By co-transfection of 293FT producer cell line with pLP-plasmids encoding for viral structural proteins, polymerase and reverse transcriptase (pLP/VSVG, pLP-1, pLP-2) and pLenti6/V5-plasmids containing GFP or CD40L at the multiple cloning sites lentiviral particles were generated. In preliminary projects the human CD40L cDNA was amplified from pcDL-SRalphahCD40L by PCR and cloned in pLenti6/V5-D-TOPO vector (Figure 8). Seventytwo hours after co-transfection the supernatant was harvested and 100-fold up-concentrated by ultracentrifugation. Ultracentrifugation was performed using Sorvall RC 5C PLUS superspeed centrifuge and fixed angle TFT 8013 rotor(Sorvall Products, L.P. Newtown, Connecticut, USA) at 103 600xg, at 4°C for 90 minutes, resuspended in CellGro and stored at -80°C until further use for maximum 6 month.

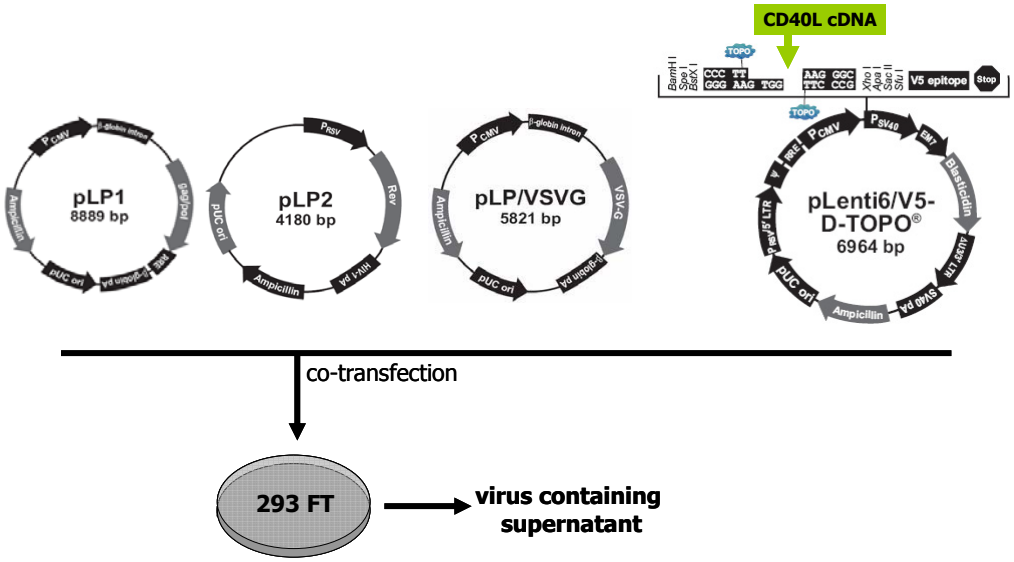

Figure 8|Co-transfection of 293FT producer cell line with three packaging plasmids (pLP1, pLP/VSVG, pLP2) and pLenti6/V4 plasmid containing CD40L or GFP cDNA leads to formation of lentiviral particles in the supernatant of 293FT cell culture.

2.4.2 Lentiviral transduction of DC

48 h and 6 h pre-matured DCs were transduced with lentiviral particles (250 µl 100x concentrated lentiviral supernatant/1x10^6 DC) in combination with 6 µg/ml Polybrene (Sigma-Aldrich,) plus IL-4, GM-CSF and IFN-γ in standard concentrations. In detail DCs were harvested after maturation with Accutase and washed once with D-PBS. For transduction with lentiviral particles 1x106 DC were centrifuged and supernatant was completely removed. DCs were resuspended in lvCD40L or lvGFP and supplemented with IL-4, GM-CSF, Polybrene and IFN-γ at standard concentrations. The cells were then transferred to a 48-well plate and incubated for 6 hours at 37°C at 5 % CO^2. After lvCD40L/lvGFP transduction the cells were detached with cell aggregate disociation medium (Accumax, Innovative Cell Technologies Inc., San Diego, CA) by application of the same volume Accumax to DC culture for 20 minutes at 37°C, washed and exact cell number was assessed by TruCount system. Then cells were used for alloMLR and separately cultured at concentration of 1x10^6 cell/cm²/ml with IL-4, GM-CSF and IFN-γ at standard conditions. For IL-12 and IL-10 quality control supernatant was taken 24 h after transduction with lvCD40L/lvGFP and expression of GFP/CD40L and phenotypic markers CD80, CD86 and MHC II were measured after 48 h following standard procedures.

2.4.3 Datamining lentiviral titer

HeLa cell line was used to assess lentiviral titer by plating $5x10^5$ cells in 6-well plates for 12-15 hours and then transduced with 10^{-2} to 10^{-10} dilutions of lentiviral supernatant in RPMI, 10 % FCS in presence of 6 µg/ml Polybrene. Fourtyeight hours after transduction with lentiviral particles the culture medium was supplemented with 2 µg/ml Blasticitin (Invitrogen Corporation, Bethesda, MD to test for antibiotic resistance after transduction. Medium was changes every 1 – 2 days. During the next 3 weeks transduced single cells grow under selective pressure to colonies which could be stained by application of Türks solution (Merck, Darmstadt, Germany) directly to cell lawn for 10 min at 4°C, followed by two washing steps with 1xPBS and count to establish an average Transforming Unit (TU). All tested lentiviral supernatants comprised TUs between $7,5x10^5$ to $6x10^5$.

To assess the multiplicity of infection (MOI, defined as the number of viral particles infecting one cell) in lentiviral supernatants different TU – cell number ratios were tested by transfection of 6 h pre-matured DCs, resulting in MOI of 0,2, that was used in all further transfection procedures.

The titer obtained with HeLa cells used is expected to be approximately 10-fold lower than the titer obtained when using HT1080 cells that were recommended to use from the manufacturer (Invitrogen, ViraPower Lentiviral Expression System, instruction manual).

3 Results

3.1 Mature DCs show improved T cell co-stimulatory function and secrete IL-12

DCs were differentiated from monocytes which are derived from aphaeresis products by density centrifugation (Elutration) during culture over 6 days in CellGro medium supplemented with IL-4 and GM-CSF. Compared to adherence, elutriation generates DC precursor cells with a higher percentage of $CD14^+$ cells and increased IL-12 secretion from mDCs [58].

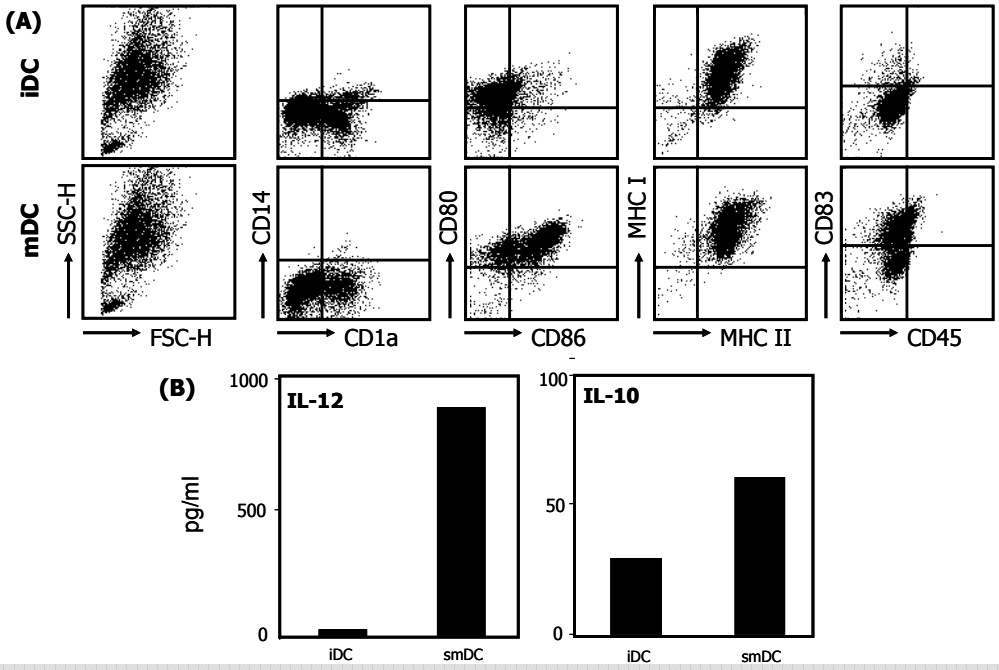

Figure 9|Maturation status of mature DCs compared to immature DCs: DCs were generated from monocytes by differentiation with IL-4 and GM-CSF during 6 days cell culture. After differentiation DCs were left untreated (iDC) or matured with LPS/IFN-γ for 6 hours (smDC). Cytokines release in supernatant was analysed 24 hours after maturation, surface molecule expression of DCs was measured 48 hours after maturation. (A) Expression pattern of surface molecules determined by flow cytometric analysis is shown. Compared to iDCs (upper panels) mDCs show up-regulation of CD80, CD86, CD83, slight increase in MHC I and II expression and down-regulation of CD14. (B) Shows cytokine expression profile of smDCs measured 24 h after triggering maturation with LPS/IFN-γ. Supernatant was analysed for IL-10 and IL-12 expression using ELISA technique. mDCs show increased expression of IL-12 and IL-10 compared to iDCs. One representative experiment out of 10 is shown.

After differentiation, DCs were matured with TLR 4 ligand Lipopolysaccharide (LPS) and the pro-inflammatory cytokine IFN-γ. Maturation for 6 hours generated semi-mature DCs (smDCs) with a peak expression of IL-12, whereas maturation for 48 hours generated fully matured DCs (mDCs), which have exhausted their capacity for IL-12 secretion. After activation of DCs the maturation status was measured by detection of the co-stimulatory molecules CD80, CD86, the activation marker CD83, CD1a, MHC I, MHC II and CD14a expressed on the surface of smDC and mDC 48 hours after treatment with LPS/IFN-γ using flow cytometric analysis (Figure 9A). Additionally, 24 hours after maturation (in detail: 6 hours maturation followed by 18 hours culture in normal culture medium) samples were taken from smDC supernatant and accumulated amounts of expressed IL-12 and IL-10 were measured by ELISA. We observed up-regulated expression of the Th1 polarising cytokine IL-12 after maturation with LPS/IFN-γ with the peak expression after 24 hours but also expression of the immune suppressive cytokine IL-10 (Figure 9B).

3.2 Lentiviral gene transfer system efficiently infect primary cells and cell lines leading to ectopic expression of CD40L

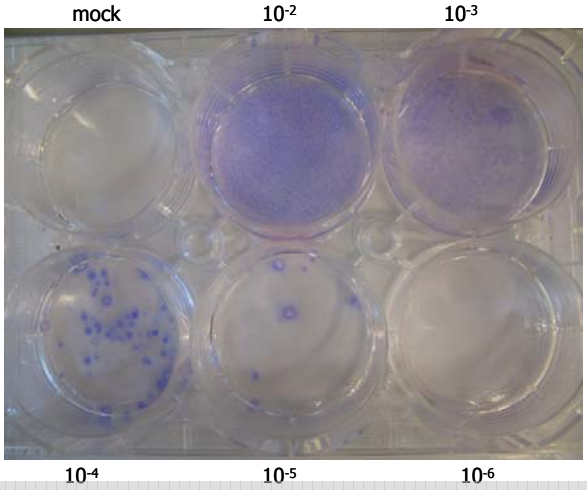

Figure 10|LV titer: HeLa cells transduced with different dilutions of lentiviral supernatant (LV-N°7) under selective conditions using blasticidin during 4 weeks cell culture are shown. Blue spots represent colonies with integrated blasticidin resistence gene located on pLenti6-V4 vector. Mock indicates un-transduced HeLa cells. TU of $8x10^5$ was generated using LV-N°7, as one representative experiment out of 3.

Lentiviral particles were produced by co-transfection of the 293FT producer cell line with plasmids encoding the structural packaging proteins, reverse transcriptase and the transgenes CD40L or GFP-control (see Figure 8, chapter 2.4.1. Generation of lentiviral particles). After 72 hours the supernatant was harvested and 100 times concentrated by ultracentrifugation. Prior to lentiviral transduction of DCs the titer was assessed by infection of the HeLa cell

line with different dilutions of lentiviral supernatant. HeLa cells were plated at 50 % confluency. The next day, lentiviral supernatant was diluted 10^{-2} to 10^{-10} in RPMI supplemented with 10 % FCS and administered to adherent HeLa cells. Forty-eight hours after transduction, the medium was changed and fresh RPMI, 10 % FCS supplemented with 2 µg/ml blasticitin was added. After four weeks of cell culture under selective conditions single cells grew to colonies, which then were counted to obtain the number of transforming units (TU) in every lentiviral batch tested (Figure 10). To define the optimal transduction protocol with sufficient transgene expression in DCs as target cells, different multiplicities of infection (MOI) were tested. Therefore, $0{,}5x10^6$ and $1x10^6$ mDCs were treated with 250 µl viral supernatant (MOI of 0,4 and 0,2, respectively) supplemented with IL-4, GM-CSF and IFN-γ. As recommended in the manufacturer's manual we used hexadimethrine bromide (Polybrene) to enhance the transduction of lentivirus into DCs. Six hours after the transduction, CellGro medium supplemented with IL-4, GM-CSF and IFN-γ was added to the DC culture to the total volume of 1 ml/$1x10^6$ DCs/cm². Supernatant was taken 24 hours after the transduction to determine the amount of cytokines expressed, whereas the expression of the transgene GFP/CD40L and the maturation status of DCs was measured 48 hours after transduction. Fifteen to 90% of viable lvCD40L-DCs showed expression of CD40L compared to un-transduced DCs with MOI=0,4 and MOI=0,2. The percentage of dead cells increased with enhanced MOI, but the percentage of GFP/CD40L positive cells remained equal (data not shown). Hence we used MOI=0,2 in CellGro culture medium supplemented with IL-4, GM-CSF, IFN-γ and Polybrene for further experiments.

3.3 Lentiviral transduction did not affect expression of DC phenotype markers

After generating lentiviral supernatant by co-transfection of the 293FT producer cell line and 100 times concentration, the titer was determined using negative selection of transduced HeLa cells. SmDCs were then transduced to compare GFP/CD40L expression of different lv-production batches and different monocyte-donors. To

compare the effect of lentiviral transduction and CD40L interaction on DCs with different maturation status we used differently pre-matured DCs.

After differentiation for 6 days with IL-4/GM-CSF, dendritic cells were matured by exposure to LPS/IFN-γ for 6 hours or 48 hours to generate smDCs and mDCs, respectively. After maturation, the cells were washed and resuspended in lentiviral supernatant (250µl lvCD40L or lvGFP/1x106 c, MOI=0,2) supplemented with IL-4, GM-CSF, IFN-γ and Polybrene. Control groups were handled the same way, including centrifugation steps, but left un-transfected (no-lv). After 24 hours, aliquots were analysed for IL-12 and IL-10 production, after 48 hours cells were harvested and analysed by flow cytometry for surface expression of phenotypic maturation markers. Ectopic expression of the transgene CD40L or GFP was measured 48 hours after transduction (Figure 11). The ectopic expression profile of the transgene did not show significant differences depending on the length of maturation. For reasons of enhanced auto-fluorescence after maturation and transduction, lvCD40L-DC were used as control for GFP expression and the other way round.

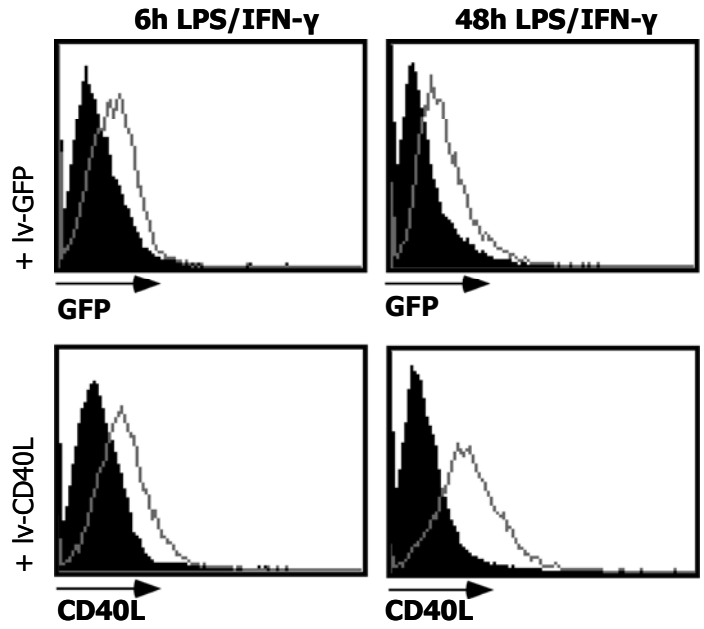

Figure 11|CD40L/GFP Expression: DCs were matured 6 hours or 48 hours with LPS/IFN-γ. Matured DCs then were transduced for 6 hours with lentiviral supernatant using MOI=0,2. Expression of CD40L or GFP on the surface of mDCs and smDCS was measured 48 hours after transduction. Blank histograms show CD40L/GFP expression of CD40L or GFP transduced DCs. Filled histograms show the transgene expression of matured transduced but unlabeled DCs. For transduction we used LV N°7; one representative out of 5 experiments is shown.

The maturation status was determined by measuring the expression of the co-stimulatory molecules CD80, CD86 and CD83 of lentivirally transduced DC. Compared to the control groups, no significant further up-regulation of phenotypic maturation molecules was observed due to lentiviral transduction or CD40/CD40L interaction (Figure 12).

We observed a full maturation status in mDCs with enhanced up-regulation of CD83 compared to smDCs. No further up-regulation of CD80 and CD86 was observed in mDCs compared to smDCs. MHC I and II surface molecule expression on lentivirally transduced smDCs and mDCs were also compared with appropriate control, but we observed no difference (data not shown).

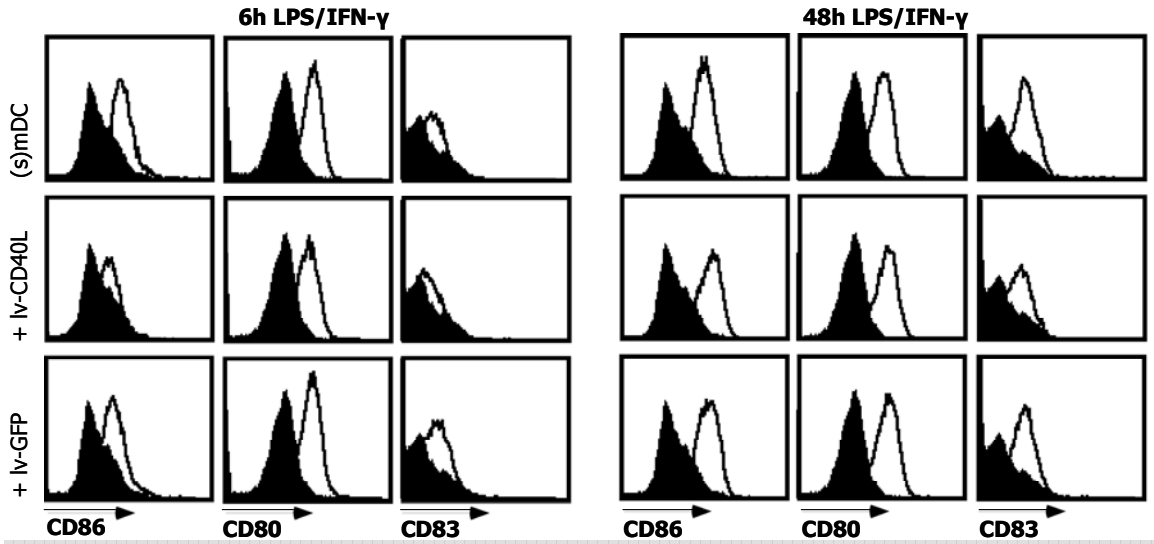

Figure 12|Maturation status of lentiviral transduced smDCs and mDCs: Maturation phenotype of 6 hours pre-matured and 48 hours pre-matured DCs after lentiviral transduction is shown. Filled histograms reflect fluorescence intensity (FI) of phenotypic markers CD86, CD80, CD83 of untransduced iDCs, whereas blank histograms show FI of phenotypic surface molecules 48 hours after transduction of smDCs and mDCs with lvCD40L and lvGFP. DCs were pre-matured for 6 or 48 h with LPS/IFN-γ and subsequent transduction was performed for 6 h with Polybrene and IFN-γ supplemented. One representative experiment out of 9 shown.

3.4 Rescue of Th1 related cytokine expression by CD40L

The cytokine expression profile provides first evidence for the high potency of CD40L as inducer of immune stimulatory cascades: although the IL-12 and IL-10 cytokine expression was increased in smDCs due to lentiviral transduction, mDCs did not respond to lentiviral gene transfer alone. Lentiviral gene transfer into 6 hours pre-matured DCs (smDCs) induced a boost of IL-12 and IL-10 cytokine expression compared to un-transduced DC control groups. Unlike smDCs expressing GFP, the ectopic expression of CD40L on smDCs further triggered an increase in IL-12 and IL-10 expression. Interestingly, mDCs, which are known to be exhausted in terms of cytokine expression, could be re-activated to express IL-12 using lv-CD40L, but no

expression of IL-10 was shown. Control DCs transfected with lentiviral particles containing genetic information to express GFP did not show re-expression of cytokines in terminally differentiated (mDCs) (Figure 13, lower diagram). The cytokine expression was measured 24 hours after the start of transduction of pre-matured DCs. These data are in agreement with preliminary data where co-cultures with CD40L expressing cell lines were performed (Dohnal et al.; unpublished observations) provides evidence that CD40L can trigger Th1 polarised second step maturation of DCs.

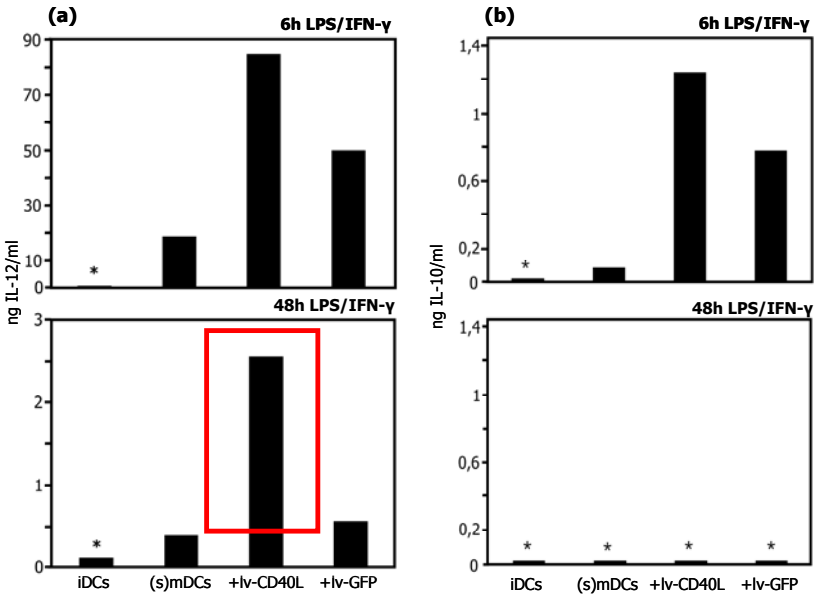

Figure 13|Cytokine secretion pattern: (A) and (B) show IL-12 and IL-10 secretion, respectively, of smDCs and mDCs compared to iDCs and transduced DCs. (A) IL-12 expression was re-initiated by lvCD40L transduction of cytokine exhausted mDCs, whereas (B) shows that no IL-10 was further expressed after lvCD40L treatment of mDCS. Amount of cytokines was measured by ELISA 24 h after transduction. Cytokine amounts below the detection limit are indicated by asterisks. One representative experiment out of 4 is shown.

3.5 Negative depletion increased purity of $CD3^+$ T cells

CD40L expressing DCs were generated in two different ways: first, smDCs were transduced with lv-CD40L which led to increased expression of IL-12 and IL-10 (lv-smDC); on the other hand, exhausted mDCs were transduced with lv-CD40L and thus re-activated for IL-12 expression (lv-mDC). To analyse the T cell stimulatory potential of these lv-DCs compared to un-transduced DCs, allogeneic mixed lymphocyte reactions (alloMLR) were performed. DCs and T cells from different

donors (allogeneic) were co-cultured for 6 days and afterwards activation and proliferation of different T cell subsets was analysed. AlloMLRs were set in DC:T cell rations from 1:3, 1:9 and 1:27 to cover a broad spectrum of the stimulation capacity of DCs. Since we were interested in proliferation of $CD4^+$ or $CD8^+$ T cells as main effector cells stimulated by DCs in the immune system, we focused on these two subsets by $CD3^+$ purification from whole peripheral blood mononuclear cells (PBMCs). To enrich $CD3^+$ T cells, we made the decision to use negative depletion, which means that $CD3^+$ target cells stay untouched and are not activated by CD3 crosslinking. Contaminating cell populations were indirectly magnetically labelled and retained in the magnetic columns leading to generation of highly pure $CD3^+$ T cells.

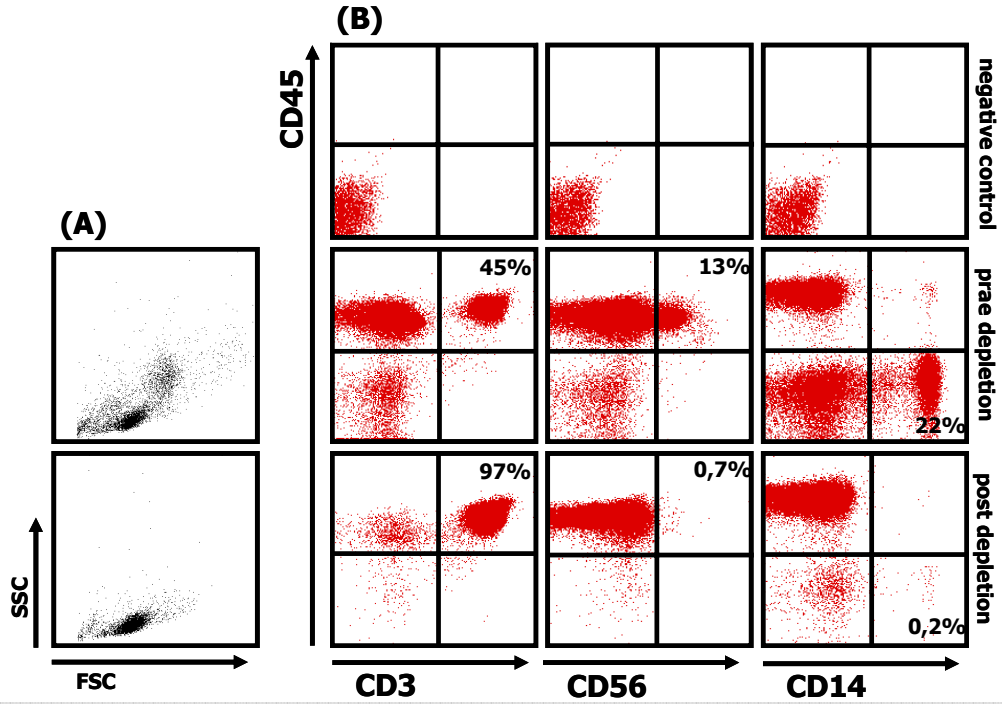

Figure 14|CD3 purified T cells used for allogeneic mixed lymphocyte reaction (alloMLR): PBMCs were indirectly magnetically labeled and retained to magnetic field by using anti-CD14, CD19, CD36, CD56, CD123 and CD235a antibodies (A) Distribution of cells on the basis of cell size (FSC) and granulation (SSC) is shown before and after CD3 purification. (B) Percentage of contaminating cell polulations is shown compared to negative un-labeled control. Reduction of $CD56^+$ NK cells and $CD14^+$ monocytes was achieved by negative depletion of PBMCs. Data analysis was performed using flow cytometric analysis. One representative experiment out of 7 is shown.

Before and after depletion the cells were analysed by flow cytometry and the percentage of $CD3^+$ T cells was compared to the percentage of contaminating non-T cell populations like monocytes, macrophages, B cells, natural killer (NK) cells, and erythroid cells (CD14, CD16, CD19, CD36, CD56, CD123 and CD235a) was compared.

The amount of $CD3^+$, $CD56^+$ NK cells and $CD14^+$ monocytes left after depletion is shown in Figure 14.

The distribution on the basis of cell size (forward scatter, FSC) and granulation (sideward scatter, SSC) shows the whole cell population before and after depletion and indicates the retained non-T cell populations after effective CD3 purification (Figure 14A). The percentage of $CD14^+$ cells in the post-depletion sample indicates that nearly all monocytes were retained in the column and the total amount of monocyte contamination was reduced to 0,2 % $CD14^+$ of total cells (Figure 14B). The amount of $CD56^+$ cells was decreased from 13% in PBMCs to 0,7 % of total cells. We obtained a purity of 97 % $CD3^+$ T cells (Figure 14B). All CD3 purifications performed obtained purities ranging from 71 % to 97 % of $CD3^+$ T cells in total cell population (Figure 14 shows one representative out of 7 experiments).
After purification, $CD3^+$ T cells were labelled using carboxyfluorescein succinimidyl ester (CFSE) to measure T cell proliferation. CFSE is taken up by T cells and diluted into progeny cells by cell division. Before purifying $CD3^+$ T cells, DCs were differentiated from monocytes using IL-4, GM-CSF during 7 days culture. On day 5, one part of these DCs was matured by TLR4 ligation with LPS/IFN-γ for 48 hours, the other part was LPS/IFN-γ matured on day 7 for 6 hours. After maturation, the cells were harvested, washed and transduced with lv-CD40L or lv-GFP at MOI of 0,2 for 6 hours in CellGro medium supplemented with IL-4, GM-CSF, IFN-γ and Polybrene. To evade cluster formation cells were then harvested using cell aggregate dissociation medium (Accumax, Innovative Cell Technologies Inc., San Diego, CA) and a single cell suspension was re-covered. We observed tight cell-cell contact in lv-DCs after transduction, which could not be disrupted by conventional Accutase treatment or resuspension.
$CD3^+$ CFSE labelled T cells were then co-cultivated with different lv-DC numbers, ranging from 1:1=DC : T cell ratio to 1:27 in alloMLR for 6 days. As control for maximum proliferation, $CD3^+$ T cells were incubated with staphylococcal exotoxins A and B (SEA/SEB). Background proliferation of T cells was shown in culture medium alone. The maturation phenotype, the percentage of ectopically expressed GFP or CD40L and cytokine expression was measured as quality control of lentiviral transduction. Therefore lentivirally transduced DCs retained after transduction from

alloMLR were cultured at final concentration of $1x10^6$ DCs/ml/cm² in CellGro medium supplemented with IL-4, GM-CSF and IFN-γ. Supernatant was analysed 24 hours after transduction for cytokine expression, maturation phenotype and ectopic expression of the transgenes GFP or CD40L after 48 hours.

3.6 LvCD40L-mDC stimulation decreases percentage of T cells with intermediate expression of CD25

After 6 days, cells co-cultivated in alloMLR were harvested. Analysis of T cell expansion was performed by measuring the percentage of CFSE negative $CD4^+$ and $CD8^+$ T cells using flow cytometry. Further on, the proliferation of $CD3^+$, $V\delta2^+$ and Granzyme B^+ (GrB) T cells was measured. The CD25 surface molecule expression indicated the activation status and the possible effector function of T cells.

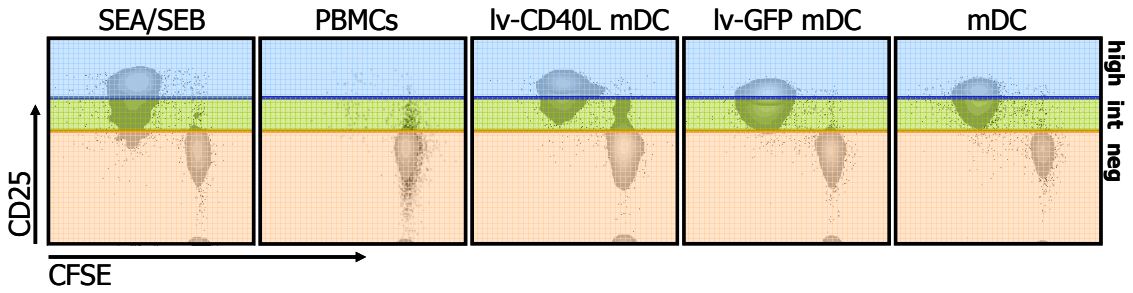

Figure 15|Discrimination of activated $CD4^+$ T cells by CD25 expression: CD3 purified T cells were co-cultivated with lentivirally transduced DCs and control un-transduced DCs in different ratios. 1:27=DC:T cell ratio of alloMLR measured on day 6 is shown compared to SEA/SEB positve control and un-stimulated PBMCs only. $CD4^+$ T cells, activated with GFP or CD40L expressing mDCs or only mDCs are shown. Lentiviral transduction of CD40L compared to GFP indicates different CD25 distributions in total $CD4^+$ T cell population. One respective experiment out of 5 is shown.

This activation status of T cell subpopulations was thereby used to discriminate between putative T helper (Th) cells as $CD4^+CD25^{int}$ and $CD4^+CD25^{high}$ regulatory T cells (T_{reg}) (Figure 15). After alloMLR we observed the expansion of two populations of $CD4^+$ T cells expressing CD25 in different surface densities. For detection of expanding activated T cells we analysed CFSE negative $CD25^+$ T cells, which generated two populations with high and intermediate amounts of CD25 expressed on cell surface ($CD25^{high}$ and $CD25^{int}$, respectively; Figure 15). Cut off levels of $CD25^{neg}$, $CD25^{int}$ and $CD25^{high}$ expression was always compared to

appropriate negative (only $CD3^+$ T cells cultured in medium only; PBMCs) and positive control (SEA/SEB stimulated $CD3^+$ T cells).

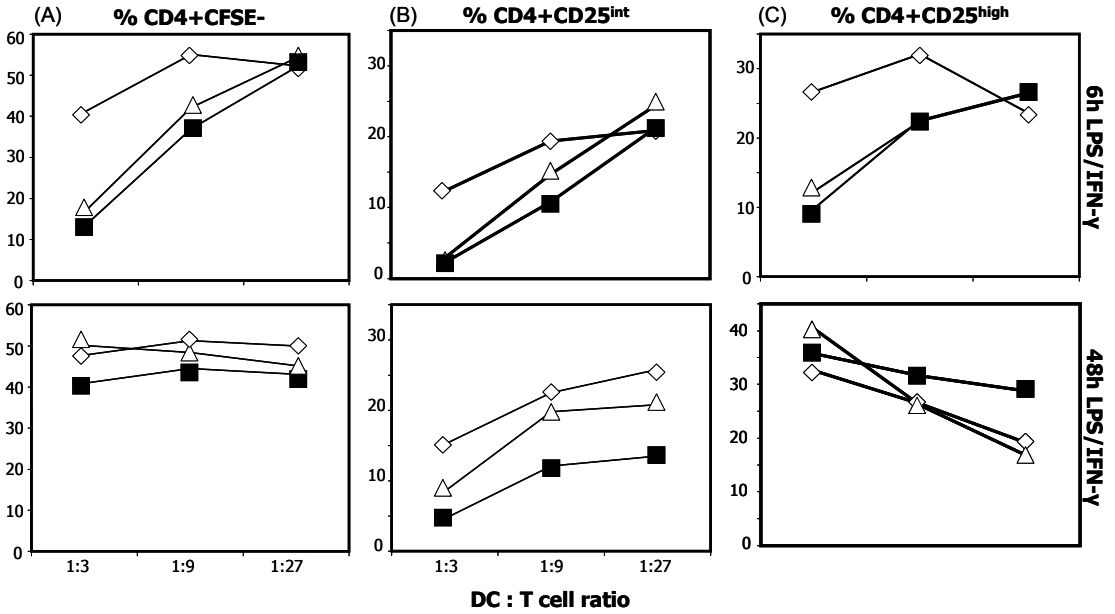

Figure 16|Proliferation and activation of $CD4^+$ T cells in alloMLR: $CD4^+$ T cells stimulated in alloMLR after 6 days with lentiviral transduced GFP or CD40L expressing pre-matured DCs and control groups are shown. (A) Proliferation of total $CD4^+$ T cells indicate tendency of little stimulation potential triggered by lvCD40L-mDC. (B) Percentage of $CD4^+CD25^{int}$ cell population of total $CD3^+$ T cells is shown. Lv-CD40L transduction decreases stimulation potential of mDCs. (C) Percentage of highly activated $CD4^+CD25^{high}$ T cells is shown. Percentage positve cells increases when stimulated with lvCD40L-mDC compared to control groups. One representative experiment out of 5 is shown. (LvCD40L-DC = squares, lvGFP-DCs = triangles, untransduced control DCs = diamond)

The analysis of alloMLR with lv-CD40L transduced DCs revealed that no significant differences between lvCD40L-DC and control groups in stimulation capacity of total $CD4^+$ T cell population was observed, neither with 6 hours or 48 hours activation stimuli prior to transduction (Figure 16). Repeatedly we observed a tendency that CD40L signal on mDCs inhibits $CD4^+$ T cell proliferation compared to GFP expressing mDCs or un-transduced mDCs (Figure 16, lower diagram). Furthermore $CD4^+$ T cells were analysed based on the activation status by CD25 expression (Figure 16B, Figure 16C). In $CD4^+CD25^{int}$ cell populations smDCs showed again no significant differences after lv-transduction compared to un-transduced DCs, whereas lvCD40L-mDC appeared to trigger little proliferation of $CD4^+CD25^{int}$ compared to control groups (Figure 16). Highly activated $CD4^+CD25^{high}$ indicated stimulation from lvCD40L-mDCs, but lv-smDCs showed less proliferation potential than un-transduced control groups (Figure 16A).

3.7 lvCD40L-mDC enforces lytic capacity of CTLs

The potential of lentivirally transduced CD40L or GFP expressing DCs to stimulate T cell activation and proliferation was measured by analysis of $CD4^+$ and $CD8^+$ T cell expansion in an alloMLR after 6 days. As general read-out, the proliferation of $CD8^+$ T cell in all CD3 purified lymphocytes was measured using CFSE dilution in total $CD8^+$ T cells. In contrast to proliferation of total $CD4^+$ T cells (Figure 16A), lvCD40L-mDC showed enhanced stimulation capacity in $CD8^+CFSE^-$ T cell populations compared to control groups (Figure 17, lower diagram). Again no significant differences in proliferation of $CD8^+$ T cells stimulated by lentivirally transduced smDCs and control un-transduced smDCs was observed (Figure 17A, upper diagram).

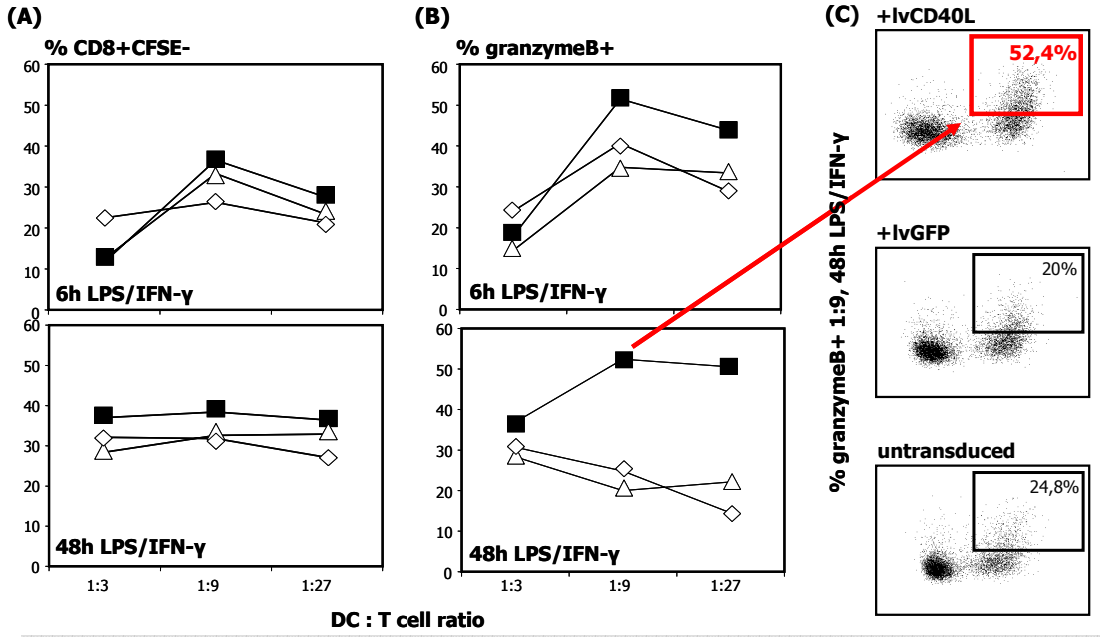

Figure 17|Proliferation and lysis capacity of $CD8^+$ T cells in alloMLR: Different DC to T cell ratios of alloMLR are shown. (A) Percentage proliferating $CD8^+$ T cells stimulated by CD40L or GFP expressing smDCs and mDCs compared to un-transduced control groups is shown. (B) Percentage of Granzyme B expressing $CD8^+$ T cells is shown. (C) Primary data of 1:9 DC:Tcell ratio of lvCD40L-mDCs indicates increased expansion of GrB^+ CTLs after stimulation with CD40L expressing mDCs in alloMLR. One representative experiment out of 5 is shown. (LvCD40L-DC = squares, lvGFP-DCs = triangles, untransduced control DCs = diamond)

Effector cell function was measured in $CD8^+$ T cells by expression level of Granzyme B (GrB) as a component of cytolytic granules within CTLs (Figure 17B). LvCD40L-smDCs showed a tendency to increase the percentage of $CD8^+GrB^+$ T cells compared to control groups, and lvCD40L-mDCs showed significant up-regulation of GrB

expression up to 2 fold compared to lvGFP-mDC and un-transduced mDC control stimulations (Figure 17C).

4 Discussion

Based on general immunological principles we set up our working hypothesis. We suggest that antigen processing DCs are matured in peripheral tissue by encountering so-called danger signals (PAMPs, pro-inflammatory cytokines), start migrating into the lymph node where they get in contact with naive T cells in a fully formed maturation status. With up-regulated co-stimulatory molecules and exogenous antigen bound to MHC II DCs they interact with the TCR and CD28 on $CD4^+$ T h cells. These Th cells start to up-regulate CD40L, that further binds to CD40 expressed on surface of matured DCs. CD40-CD40L ligation induces the second maturation step and re-initiates IL-12 cytokine secretion in mDCs, which have exhausted their capacity for cytokine secretion [10]. Also CD40-CD40L interaction triggers licensing of DCs to cross-present exogenous antigen in MHC I context to stimulate $CD8^+$ T cells. The $CD8^+$ T cells now are fully activated to function as antigen-specific CTLs.

4.1 Lentiviral transduction as an optimal method for gene transfer into resting primary cells

DCs as representatives of resting primary cells are known for the difficulties of gene transfer. The literature indicates the need for efficient gene transfer into DCs to alter the diverse immune modulatory functions of these APCs as they play pivotal roles in the immune system and therefore are interesting targets for medical application [13]. Different ways to transfer genetic information into primary not-dividing cells like DCs was found in the literature, but side-effects like increased number of apoptotic DCs and maturation initiation by using electroporation [59], the need for cell-division in retroviral genetransfer or short dated expression of transgene by transfection are common. Although transfection of DCs with adenoviral constructs is more often used [60-63] it does not result in stable integration into the genome. Therefore alternative gene-transfer methods are in demand. With lentiviral particles a continuous expression of transgene due to stable integration into host cell genome using long

terminal repeats (LTRs) can be obtained. Additionally lentiviral constructs are known to transduce resting, un-dividing primary cells [60, 62, 63] and stably express target genes.

To generate more potent Th1 polarising DCs as improvements of next generation tumour vaccines the effect of CD40L on DCs was measured by DNA micro array-based whole genome expression profiling (Dohnal et al., unpublished data) in preliminary projects. These data provided first evidence that CD40L acts as second maturation stimulus on mDCs after TLR4 activation. CD40-CD40L ligation restores the immune stimulatory capacity of terminally differentiated mDCs by re-expression of IL-12, which is a key cytokine for Th1 polarisation of immune reactions. We used CD40L and GFP expressing plasmids for co-transfection of the producer cell line 293FT with plasmids containing structural information and reverse transcriptase function to obtain lentiviral supernatant. After 100 times up-concentration by ultracentrifugation the titer was measured by negative selection of transduced HeLa cells. Generation of lentiviral particles showed to be a highly sensitive system with different critical points. High purity of plasmids for co-transfection and logarithmic growth rate of 293FT producer cell line was pivotal for production of moderate amounts of lentiviral particles in supernatant, but still the lentiviral titer generated by negative selection comprised 10^3x less transforming units (TU) than expected. Though the manufacturers' protocol indicates that higher titer numbers may be obtained by using HT1080 we only generated TUs of 7-8x10^5/ml after ultracentrifugation and concentration by factor 10^2 (Figure 9). As multiplicities of infection (MOI) values of 0,2 were used to obtain more than 50% of the transduced cells positive for transgene expression, the generated TUs are only values for comparative evaluation of different lentiviral production series but did not generate real number of lentiviral particles per ml. To assess the titer of lentiviral supernatant application of the antibiotic Blasticitin was used to select for transduced single cells that integrated after transduction a cDNA for antibiotic resistance. This selection procedure acts more crucial on HeLa cells with low lentiviral particles integrated and thus with less antibiotic resistance. These cells are deleted early after transduction and do not grow to colonies.

4.2 Maturation reduces sensitivity of DCs to genetic manipulation

In preliminary tests we also tried to transduce monocytes with lvCD40L and lvGFP to generate 100% CD40L/GFP positive DCs during 6 days differentiation culture under selective conditions. Monocytes showed to be very sensitive for lentiviral transduction as a number of apoptotic cells increased compared to un-transduced control groups. An additional maturation stimulus was triggered by lentiviral transduction prior to full differentiation, presumably by early stimulation of TLR3 with free dsRNA, derived from the lentivirus construct that resulted in un-specific differentiation to non-DC populations (data not shown).

Also fully differentiated iDCs were transfected with lentiviral supernatant to generate CD40L expressing iDCs capable of early stimulation by CD40-CD40L interaction after maturation. iDCs were shown to be very sensitive to transduction indicated by high loss of cells due to apoptosis, characterized by Pi positive cells after 48 hours post-transfection (data not shown). Remaining PI negative cells up-regulated CD80, CD86, CD83, MHC I and II and secreted IL-12, all of which showed that transfection of iDCs alone already had great potential for maturation. In contrast, pre-matured (either 6 hours or 48 hours LPS/IFN-γ) DCs showed no differences in maturation-dependent surface molecule up-regulation compared to control groups. Again, iDCs reacted to dsRNA in the cytoplasm, derived from lentiviral based gene transfer. Preliminary DNA micro array-based whole genome expression profiling that was performed prior to this project in our laboratory already indicated that TLR3 is highly up-regulated in DCs in an immature state. It was shown that the TLR4 signal transduction pathway is down-regulated after TLR4 induction. After maturation by TLR4 signalling, expression of TLR3 and down-stream signal transduction molecules was slightly down modulated.

The maturation of DCs optimised the survival of transduced cells and showed no side effects in differentiation. To determine the effect of CD40L on matured DCs we compared transduction of 6 hours pre-matured DCs (lv-smDCs) with transduction of mDCs (lv-mDCs) after 48 hours LPS/IFN-γ stimulus. Interestingly, the CD40-CD40L interaction re-initiated IL-12 expression in mDCs, which produced data that was in

conflict with the general paradigm that mDCs are terminally exhausted for their capacity to express IL-12 [10].

The CD40L interaction after LPS/IFN-γ maturation with highly up-regulated CD40 on DCs results in re-initiation of IL-12 expression, depending on the maturation state of DCs. This substantiates our working hypothesis that CD40-CD40L interaction occurs *in vivo* after maturation and triggers a second boost of DC-stimulation leading to IL-12 expression.

4.3 CD40L expressing mDCs highly activate $CD4^+$ T cells but lead to the loss of the Th cell population

In our alloMLR the potential of lv-DCs to stimulate T cell proliferation and activation was mainly measured by up-regulation of the activation marker CD25 expressed on the cell surface of $CD4^+$ T cells and the loss of CFSE fluorescence intensity of cells due to division. The general activation status of $CD4^+$ T cell is used to measure the effector function, as suppressive T cell populations expressing $CD4^+$ T cell co-receptor show high amounts of IL-2 receptor molecule CD25 presented on cell surface [64].

The expression of transcription CD25 was measured in $CD4^+$ T cells to identify T cell subsets with suppressive or regulatory function. Transduction of mDC with lv-CD40L showed increase of $CD4^+CD25^{high}$ cell population.

The detection of the forkhead family transcription factor 3 (FoxP3) expression was originally used to identify suppressive T_{reg} cells after *in vitro* expansion. Recent data suggests that FoxP3 acts as activation marker that is transiently up-regulated in $CD4^+CD25^{high}$ T cells with no suppressive function. Only naturally occurring T_{reg} cells show stable expression of FoxP3 [65, 66]. Recent data provide evidence that epigenetic modifications of the translational region of the FoxP3 gene locus are related to stable FoxP3 expression only in T_{reg} cells [65]. Detection of the FoxP3 protein is no indicator for suppressive or regulatory function of $CD4^+CD25^{high}$ T cells. In contrary $CD4^+$ Th and T_{reg} cells can be distinguished by analysis of CD25 expression (Figure 15). The IL-2 receptor molecule CD25 is highly up-regulated in T_{reg} cells whereas activated Th cells only express CD25 in moderate amounts [67].

One function of highly up-regulated CD25 might be that IL-2 as proliferation supporting cytokine is depleted from the surroundings by being captured in high amounts by CD25 molecules expressed on T_{reg} cells [68]. Another model suggests that T_{reg} cells act cell-contact dependent and highly up-regulated CD25 surface molecules only indicate expansion triggered by IL-2. The helper or regulatory function of $CD4^+CD25^+$ populations may only be analysed by the effect of these cells on other T cell subsets in co-cultures.

In alloMLR CD40L expression on mDCs stimulated $CD4^+CD25^{high}$ T cell expansion but only decreased numbers of $CD4^+CD25^{int}$ expressing subpopulations, the later with potential Th cell function (Figure 16), were measured. These findings provide first evidence that ectopically expressed CD40L on DCs adds the licensing signal for cross-presentation of exogenous antigens in MHC I context and decreases the need for interaction with Th cells *in vitro*. The binding of CD40 to CD40L, both expressed on the DCs themselves, may capture all free CD40 molecules and therefore CD40L on activated Th cells can not be bound. This then may lead to decreased Th proliferation. Th cell independence has to be investigated by further experiments using CD4 and CD8-purified T cells in alloMLR reaction with lvCD40L-mDCs.

4.4 Cytolytic activity of CTLs is IL-12 dependent

The T cell activation analysis of $CD8^+$ CTLs after 6 days of alloMLR reaction was performed by measuring decrease of CFSE fluorescence intensity due to proliferation. The cytolytic function of $CD8^+$ T cells was analysed by expression of Granzyme B (GrB) as key component of cytolytic granules of activated CTLs that is critical for T cell receptor-induced cell death [69]. The activation potential of lvCD40L-mDCs in alloMLR provided first evidence that IL-12 exhausted mDCs with re-initiation of IL-12 production due to CD40L transduction have increased CTL priming capacities compared to control groups (Figure 17).

We conclude that these findings have the potential to impact on the design of DC based cancer vaccines.

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